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The Mechanism of Action of Aspartase.

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THE MECHANISM OF ACTION OF ASPARTASE

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ABSTRACT

The mechanism of the reaction catalyzed by aspartase (L-aspartate ammonia-lyase, EC 4.3.1.1) from Enterobacter aerogenes was investigated at pH 7.0 in the presence of 1 mM Mg^{2+} by four major kinetic methods. These included initial velocity, product inhibition, and isotope effect studies as well as isotope exchange studies under conditions of chemical equilibrium and non-equilibrium. Also, the addition of chromatography on columns of Sephadex G-200, hydroxylapatite, and DEAE-Sephadex A-50 to the earlier purification scheme of Williams and Lartigue (1967, 1969) resulted in aspartase preparations of increased purity that were free from fumarase.

The product inhibition and initial velocity patterns for the forward and reverse reactions, respectively, were consistent with a random sequential mechanism in which all steps prior to the interconversion of the central complexes were in rapid equilibrium. Apparent kinetic constants for 2S-aspartate, NH_4^+, and fumarate were determined from these patterns. The values obtained were: For 2S-aspartate, K_m = 2.0 mM; for NH_4^+, $K_m \simeq K_d$ = 63.0 mM, and K_i = 56.9 mM; for fumarate, $K_m \simeq K_d$ = 0.1 mM, and K_i = 0.089 mM. The equivalency of the apparent Michaelis and dissociation constants for NH_4^+ or fumarate implied that the binding of one reactant was independent of the presence of the other in the direction of fumarate amination.

Enzymatically-prepared forms of 2S-aspartic acid labeled with ^2H, ^3H, or ^{15}N were used in the isotope effect studies in an effort to locate the rate-limiting step of the overall reaction. The isotope

enrichment at stereospecific locations in these labeled acids was found to be essentially complete by nuclear magnetic resonance and mass spectroscopy. As a result, the determination of isotope effects from a direct comparison of the initial rates of deamination of the labeled and unlabeled forms of 2S-aspartate as substrates was justified. No primary isotope effect was observed when the initial deamination rates of 2S,3R-[3-^{2}H]aspartate and unlabeled 2S-aspartate were compared. Unfortunately, with 2S-[2-^{15}N]aspartate, the results were inconclusive. However, a secondary isotope effect of 1.11 $\pm$ 0.02 was obtained with 2S-[2-^{2}H]aspartate. Moreover, during deamination of 2S-[2-^{3}H]aspartate, a similar effect was ascertained from an increase in the specific radioactivity of the ^{3}H-labeled fumarate produced early in the reaction. These observations were consistent with the rate-limiting step of the reaction being either C-N bond breakage or release of one of the products.

The findings from a study of the exchange of the β proton of 2S-aspartate in tritiated water while the deamination reaction was proceeding indicated that the sole route of ^{3}H incorporation into 2S-aspartate was through the amination of fumarate. Very likely, the proton abstracted from the aspartate was released from the enzyme subsequent to C-N bond breakage. Under differing sets of equilibrium concentrations of reactants, the exchange of ^{15}N between [^{15}N]NH_4^+ and aspartate was found to be 20 to 38% more rapid than the corresponding exchange of ^{14}C between [1,4-^{14}C]fumarate and aspartate. In order to account for this faster ^{15}N exchange, C-N bond breakage had to be fairly rapid with

respect to the dissociation of fumarate from the enzyme. Therefore, it was concluded that the rate-limiting step of the aspartase-catalyzed reaction was the release of either fumarate or the abstracted proton from the enzyme surface.

I. LITERATURE REVIEW

Introduction and Objectives

Aspartase (L-aspartate ammonia-lyase, EC 4.3.1.1) catalyzes the conversion of 2S-aspartate (equivalent to L-aspartate) to fumarate and NH_4^+ by the following reversible reaction:

$$^-OOCCH_2\text{-}\underset{\displaystyle NH_3^+}{\underset{|}{CH}}COO^- \rightleftharpoons {}^-OOCCH{=}CHCOO^- + NH_4^+$$

[illegible] of the enzyme was first postulated by Harden (1901), and the stoichiometry of the above reaction was later established by Quastel and Woolf (1926).

Aspartase is now recognized as just one of a number of enzymes that catalyze the removal of HX from adjacent carbon atoms of substrate molecules in elimination reactions of the type:

$$H\text{-}\overset{|}{\underset{|}{C}}\text{-}\overset{|}{\underset{|}{C}}\text{-}X \rightleftharpoons \diagdown\,\diagup C{=}C\,\diagup\diagdown + HX$$

The leaving group X is usually an amino or hydroxyl moiety, but it can be of a more complex nature, such as AMP[1]. Those enzymes that effect the removal of ammonia from certain amino acids, giving the corresponding olefinic acids, are called amino acid ammonia-lyases. Representatives of this group include β-methylaspartase, histidase, and phenylalanine ammonia-lyase, in addition to aspartase. Enzymes that catalyze the

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[1]For the definitions of abbreviations, refer to Appendices I and II.

elimination of water from their substrates are called hydro-lyases. One well-known example is fumarase which converts 2S-malate to fumarate. These various elimination reactions have usually been described as β-eliminations in which the β carbon contains the proton abstracted. It is convenient to refer to the carbon bonded to the leaving group X as the α carbon. In the case of aspartate, the α and β carbons correspond to carbons 2 and 3, respectively.

The distribution of aspartase has been reviewed by Erkama and Virtanen (1951); Virtanen and Ellfolk (1956); Williams and Lartigue (1969). Although aspartase occurs mainly in bacteria and a few species of higher plants, its presence in certain animal tissues has been suggested. Kurata (1962) found aspartase activity in homogenates from the ontogeny of the frog Rhacophorus schlegelii. In a recent article, Cutinelli et al.(1972) reported a similar activity associated with the mitochondrial fraction from the liver of certain elasmobranch fishes.

The enzyme has been regarded primarily as a catabolic enzyme in the amino-acid metabolic schemes of various kinds of bacteria and plants. Vender et al. (1965) and Marcus and Halpern (1969) have proposed that when glutamate-utilizing cells of E. coli are grown on glutamate as the sole carbon source, the glutamate is converted via transamination with oxaloacetate to α-ketoglutarate and aspartate. The aspartate is subsequently deaminated to fumarate which then can be transformed back to oxaloacetate. However, unlike many catabolic enzymes, the aspartase reaction is readily reversible, and its equilibrium constant actually favors aspartate formation (Bada and Miller, 1968). This, together with the allosteric behavior of the enzyme (Williams and Lartigue, 1967, 1969; Rudolph and Fromm, 1971; Suzuki et al., 1973), suggests that aspartase may be a regulatory enzyme

that could function synthetically, particularly under conditions in which aspartate is removed. Vender and Rickenberg (1964) have reported findings indicating that even though NH_4^+ assimilation in E. coli is catalyzed predominantly by an NADP-linked glutamate dehydrogenase, amino acid formation can also occur via the amination of fumarate. Moreover, Tosa et al. (1973) have developed a process allowing the continuous synthesis of 2S-aspartate with 95% yield by the passage of ammonium fumarate through a column of aspartase immobilized in a polyacrylamide gel lattice.

The steps involved in the mechanism of the reversible, non-enzymatic deamination of 2RS-aspartic acid in the presence of base have been studied in detail by Bada and Miller (1970). Consequently, it was of interest to determine if aspartase catalyzed aspartate deamination by a similar mechanism as proposed for the non-enzymatic case. First, however, the sequence by which the enzyme binds 2S-aspartate and releases the resulting NH_4^+ and fumarate has to be known in order to better understand the chemical events taking place at the enzyme's active site. Such a sequence and any additional characteristics of substrate binding and product release constitute the kinetic mechanism of aspartase. An extensive review of the literature revealed that only the stereochemical course of the reaction had been determined with certainty, in spite of the availability of several excellent reviews about aspartase (Virtanen and Ellfolk, 1956; Ellfolk, 1956; Williams and Lartigue, 1969). However, knowledge of the reaction's stereochemistry provides little information concerning the steps in the chemical mechanism. Furthermore, a kinetic scheme for the reaction had not been proposed in the literature. Therefore, investigations were immediately undertaken in this laboratory for purposes of elucidating the kinetic scheme of the apartase reaction and

obtaining information about the accompanying chemical mechanism.

The activity of aspartase has been determined by measuring either the production of NH_4^+ or fumarate upon incubation of the enzyme with 2S-aspartate or the appearance of 2S-aspartate in a medium containing fumarate, NH_4^+, and enzyme. The most convenient method is to follow the continuous production of fumarate spectrophotometrically at 240 nm (Williams and Lartigue, 1967). As would be expected, this method requires that the enzyme preparations be essentially free of other enzymes that may utilize 2S-aspartate or fumarate or substrates. In this respect, the presence of fumarase is particularly troublesome. Williams and Lartigue (1967, 1969) developed a purification procedure that yielded aspartase preparations from Enterobacter aerogenes which were claimed to be free of fumarase and other enzymes that might complicate the study of the enzyme. Although their preparations were not homogeneous with respect to aspartase, the purity was the highest yet reported at that time. Since then, improvements in the procedure have been made in this laboratory giving preparations of increased specific activities. However, some of the preparations were unexpectedly found to be contaminated with fumarase. Because several of the planned mechanistic studies involved lengthy incubation periods, it was necessary to include in the purification scheme a step whereby fumarase could be conveniently and completely removed as a contaminant.

The present purification procedure as well as studies of the aspartase reaction mechanism through 1972 have been summarized by Dougherty et al. (1972). In addition, these mechanistic aspects and other properties of the enzyme have been included in a comprehensive review of the enzymatic elimination of ammonia (Hanson and Havir, 1972). Therefore, only litera-

ture pertinent to the investigations presented in this dissertation or appearing subsequent to 1972 will be discussed throughout the remainder of this chapter.

Purification and Physical Properties of Aspartase

Maintenance of reasonable levels of activity of purified aspartase preparations during weekly storage periods at 4° has always been a constant problem. The enzyme prepared from Enterobacter aerogenes by Williams and Lartigue (1967, 1969) was no exception. The fractions obtained following chromatography on DEAE-cellulose, the final step in their scheme, was quite unstable. As a result, experiments with the enzyme had to be done as soon as possible. The deterioration upon storage appeared to be irreversible, for attempts to reactivate such denatured preparations by incubation with various thiol compounds were futile. On the other hand, the activity of crude fractions obtained after the second $(NH_4)_2SO_4$ precipitation step could be maintained for at least six months to a year by freezing. The enzyme preparations of Williams and Lartigue usually possessed a specific activity of approximately 35 units/mg protein. Usually, one unit of aspartase is defined as that quantity which will produce one μmole of fumarate per min at pH 7.0 and 28°-30°.

Workers in this and other laboratories have attempted to improve the yield, purity, and stability of aspartase. Williams and Scott (1968) added chromatography on DEAE-Sephadex A-50 to the earlier scheme of Williams and Lartigue, but little increase in purity was effected. Using an assay based upon the amination reaction, Korobeinik and Domaradskii (1968) followed the purification of an aspartase from Pasteurella pestis. Their preparations were likewise unstable, the activity falling steeply

after five to six days of storage at -10°. In contrast, Rudolph and Fromm (1971) found that aspartase isolated from Escherichia coli, strain B, was very stable. Furthermore, their preparations were apparently homogeneous as shown by ultracentrifugation and analytical disc-gel electrophoresis. It may be worth noting that these workers used 0.1 M HEPES buffer, pH 7.0, in their stability studies instead of the usual Tris-HCl or phosphate buffers. Recently, Suzuki et al. (1973) achieved purification of an aspartase from E. coli, strain W, which was also apparently homogeneous and reasonably stable. Little loss of activity was observed following storage of their enzyme in the presence of dithiothreitol, glycerol, and $(NH_4)_2SO_4$ at 4° for up to two weeks. Although the stability was increased, the purity of the enzymes of Rudolph and Fromm (1971) and Suzuki et al. (1973) was not substantially greater than that of the aspartase routinely obtained from E. aerogenes. Furthermore, the present procedure of Dougherty et al. (1972) gave fumarase-free preparations of sufficient stability to complete the various kinetic studies to be described later. Since the primary purpose herein was to study the enzyme's kinetic and chemical mechanism of action and not to stabilize it, no attempt was made to incorporate any parts of the other procedures during preparation of the enzyme from E. aerogenes.

The molecular weight of the native E. aerogenes enzyme was found by Williams and Lartigue (1967) to be approximately 180,000. This value had to be determined with only partially purified fractions since purified aspartase was irreversibly inactivated during prolonged density-gradient centrifugation. Upon incubation with p-mercuribenzoate, pH 7.0, the native enzyme was cleaved into four apparently homogeneous subunits (45,000 molecular weight). Regeneration of active enzyme was achieved

by incubating the subunits with 2-mercaptoethanol.

The molecular weight and subunit constitution of the E. coli enzyme was determined with highly purified preparations by both Rudolph and Fromm (1971) and Suzuki et al. (1973). The former group found a value of 170,000 using chromatography on Sephadex G-150. Accordingly, a single species of 45,000 molecular weight was observed with sodium dodecyl sulfate-urea gel electrophoresis. The latter investigators obtained a slighly higher molecular weight of 193,000 using sedimentation equilibrium analysis. They also determined the existence of four subunits (48,500 molecular weight) based upon polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

At this point, it is appropriate to compare the size and constitution of aspartase to that of β-methylaspartase and fumarase. Hsiang and Bright (1967) found that the molecular weight of active β-methylaspartase corresponded to 100,000. The native enzyme was cleaved into two subunits of 50,000 molecular weight by 8 M urea. It was, in turn, dissociated into four subunits by treatment with p-mercuribenzoate. From mesaconate binding studies in which a gel filtration technique was used and from tryptic peptide mapping studies, Wu and Williams (1968) concluded that native β-methylaspartase was a tetramer containing two types of subunits. Each promoter (dimer) contained one of each type and possessed one active site. Fumarase was more similar to aspartase in that the molecular weight of the former enzyme was 194,000 (Kanarek et al., 1964). The native form was cleaved into four identical subunits of 48,500 molecular weight by 6 M guanidine HCl. There appeared to be four binding sites per molecule (Teipel and Hill, 1968); whereas the number of substrate sites for aspartase was unclear.

Kinetic Properties of Aspartase

Earlier reports in the literature indicated that the pH optima of aspartases from various bacterial sources fell within 6.5 to 7.8 for both the deamination and the amination reactions (Lartigue, 1965). In almost all cases, phosphate buffers were used in the reaction solutions. In more recent studies, however, when buffers such as Tris (chloride or acetate) or HEPES were used, the pH optima were shifted to values ranging around 8.4-8.7 (Sekijo et al., 1965; Williams and Lartigue, 1967; Rudolph and Fromm, 1971; Suzuki et al., 1973). Since it was suggested that the secondary phosphate anion has an inhibitory effect on the aspartase reaction (Williams and McIntyre, 1955), the lower values contained in the earlier reports were most likely the result of using phosphate buffers. Other causes could have been the use of lengthy assay periods or possibly concentrations of substrates that were less than saturating.

Williams and Dougherty (1970), Rudolph and Fromm (1971), and Suzuki et al. (1973) all presented evidence that the aspartases from E. aerogenes and E. coli were insensitive to carbonyl reagents such as sodium borohydride or cyanoborohydride. The latter two groups of workers observed A_{280}/A_{260} ratios in the neighborhood of 1.76-2.0 as well as negligible absorption in the visible region. These data suggested that no organic cofactors were either involved in the catalytic process or bound to the enzyme. Yet, there was evidence that for catalysis, the enzyme required the presence of a divalent metal ion. Aspartase activity which was partially reduced as a result of prolonged dialysis in the presence of EDTA could be restored by numerous divalent

cations, namely Mg^{2+}, Ca^{2+}, Mn^{2+}, Co^{2+}, and Zn^{2+} (Ellfolk, 1956; Wilkinson and Williams, 1961; Depue and Moat, 1961). In fact the E. coli enzyme was prepared completely devoid of activity in the absence of added metal ions by exhaustive dialysis in HEPES buffer, pH 7.5 (Rudolph and Fromm, 1971). Full activity of the dialyzed enzyme could then be restored by the addition of Mg^{2+}.

Initial velocity experiments with the enzymes from E. aerogenes and E. coli revealed cooperative effects of the substrate that were pH dependent. Plots of initial velocity versus substrate concentration determined for the former enzyme at pH 7.0 in the presence of constant concentrations of K^+ and Mg^{2+} were not truly hyperbolic, but drifted upward at high substrate concentrations (Williams and Lartigue, 1967). Lineweaver-Burk plots of the reciprocal of initial velocity versus the reciprocal of substrate concentration (Lineweaver and Burk, 1934) showed clearly the negative cooperativity at high substrate concentrations which resulted in an increase in both K_m and V_{max}. At pH values above 7.5, the initial velocity-substrate concentration plots became distinctly sigmoidal. Accordingly, the double-reciprocal plots curved upward at low substrate concentrations. With E. coli aspartase, the observed nonlinearity of Lineweaver-Burk plots at alkaline values of pH also illustrated a dependence upon the Mg^{2+} concentration as long as the K^+ concentration was held constant. Lastly, Suzuki et al. (1973) demonstrated that at pH 8.6 and constant Mg^{2+} concentration, K^+ exhibited an inhibitory effect at low aspartate concentrations and an activating effect at high substrate concentrations. NH_4^+ and Li^+ also produced similar cooperative effects, but to lesser extents. In contrast, Na^+ showed little effect.

In regard to other effectors, Williams and Scott (1968) observed that with some aspartase fractions from E. aerogenes, the presence of AMP and IMP significantly reduced the departure from Michaelis-Menten kinetics at pH 8.7. However, at pH 7.0, the effect of AMP and IMP was much less pronounced. Rudolph and Fromm (1971) were unable to observe any significant effect of AMP on the initial-velocity kinetics of the E. coli enzyme. Since the pH of their studies with AMP was not clearly stated, a pH of 7.0 was assumed.

Values of K_m determined for 2S-aspartate in earlier studies were previously tabulated by Lartigue (1965). Recall that the K_m of an enzyme for a particular substrate is equal to that concentration of substrate required to give one-half of the V_{max}, and, therefore, its units are given in terms of concentration. All of the earlier K_m values were of the order of 10 to 20 mM for 2S-aspartate, regardless of the source and degree of purity of aspartase. As was the case with the optimum pH determinations, phosphate buffers were used in most of these investigations. As shown in Table I, the use of buffers other than phosphate in the more recent studies resulted in K_m values that were significantly lower. In light of the cooperative nature of the interaction between enzyme and substrate, this author felt that the recorded K_m values for the substrates were more appropriately designated as apparent constants. With the exception of the value reported by Sekijo et al. (1965), the values for 2S-aspartate varied from 1 to 4 mM at pH 7.0-7.5.

Whereas aspartase from E. coli or E. aerogenes appeared to be completely specific for 2S-aspartate and fumarate, NH_4^+ could be replaced by hydroxylamine as an alternate substrate in the amination

TABLE I

Apparent Michaelis Constants for the Indicated Substrates of Aspartase from Various Sources. Pertinent conditions from studies subsequent to those summarized by Lartigue (1965) are given in the table.

Enzyme Source	Substrate	pH	K_m (mM)	Buffer and Pertinent Conditions	Reference
Escherichia freundii	2S-aspartate	---	44.0	Michaelis buffer[c]	Sekijo *et al.* (1965)
	fumarate	---	9.0		
Enterobacter aerogenes[a]	2S-aspartate	7.0	1.5	50 mM Tris-HCl; 1 mM $MgCl_2$; 200 mM K^+; 30°	Williams and Lartigue (1967)
		7.5	5.1		
Pasteurella pestis	fumarate	7.2	48.0	66 mM phosphate; 37°	Korobeinik and Domaradskii (1968)
	NH_4Cl	7.2	70.0		
Escherichia coli[a]	2S-aspartate	7.0	4.0	100 mM HEPES; 10 mM $MgCl_2$; constant K^+; 28°	Rudolph and Fromm (1971)
		8.0	5.0		
Escherichia coli[b]	2S-aspartate	7.4	1.0	100 mM Tris-HCl; 2 mM $MgCl_2$; 30°	Suzuki *et al.* (1973)
	NH_4Cl	7.4	20.0		
	NH_2OH	7.4	5.0		

[a]Potassium aspartate was used as the substrate.

[b]Sodium aspartate was used as the substrate.

[c]This was assumed to be either Michaelis' sodium barbital or barbital-acetate buffer.

reaction catalyzed by either enzyme (Emery, 1963; Suzuki et al., 1973).

Unlike aspartase, β-methylaspartase exhibited Michaelis-Menten kinetics at all pH values studied (Hanson and Havir, 1972). At low substrate concentrations, the fumarase reaction in both directions also followed Michaelis-Menten kinetics. But at high concentrations of substrate (>5 x K_m), activation was observed (Taraszka and Alberty, 1964). The pH optimum of aspartase fell approximately midway between those of fumarase (pH 7-8) and β-methylaspartase (pH 9.7).

Stereochemistry of the Aspartase-Catalyzed Reaction

Aspartase was shown to be specific not only for the S configuration of carbon 2 of aspartate but also for the proton removed from carbon 3. For reviews, see Arigoni and Eliel (1969) and Bently (1970). The specificity of aspartase towards the prochiral center of its substrate was determined by relating the amination reaction to the corresponding hydration reaction catalyzed by fumarase. Englard (1958) and Krasna (1958) observed that when enzyme preparations from E. aerogenes and Proteus vulgaris, respectively, were incubated with fumarate and $N^2H_4^+$ in ^2H_2O, the resulting aspartate and malate were labeled with essentially one ^2H per molecule. In addition, no ^2H was introduced into the fumarate. If the reactions were not stereospecific for one of the prochiral hydrogens, the recovered fumarate would necessarily have become doubly labeled with ^2H. Englard (1958) further demonstrated that the bacterial fumarase possessed the same stereospecificity as the pig-heart enzyme.

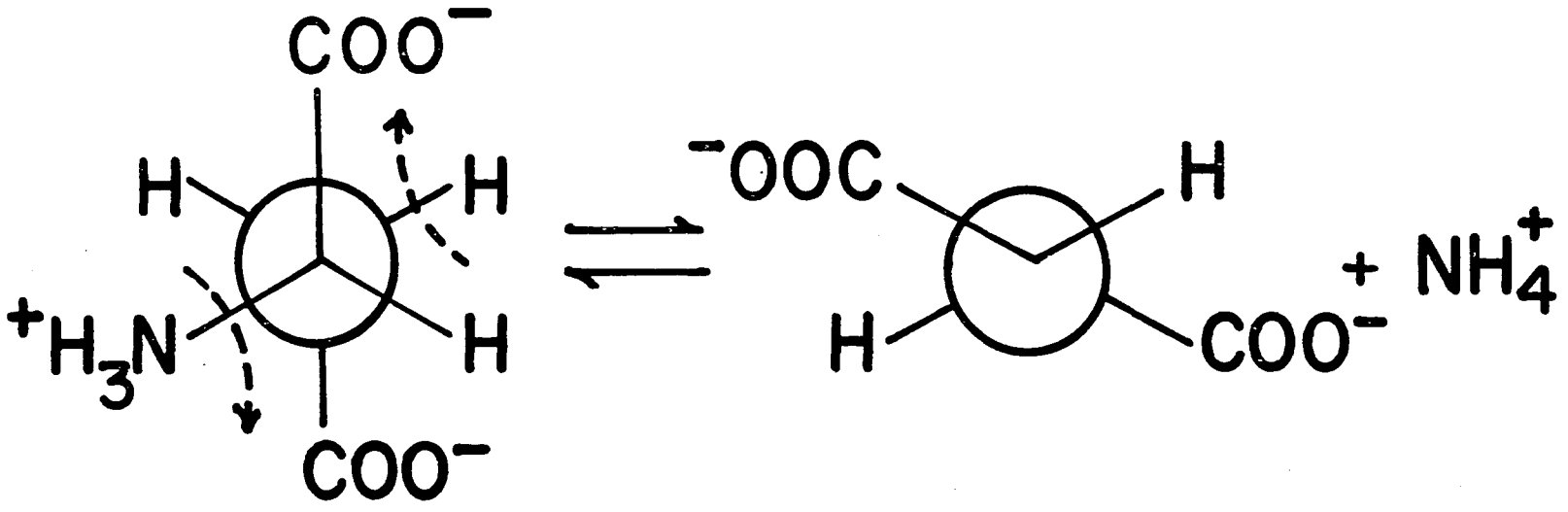
At about the same time Gawron and Fondy (1959) synthesized the racemic mixture, 2RS,3RS-[3-^{2}H]malic acid, in which the ^2H and hydroxyl

were in the threo configuration. In this configuration, the ^2H and hydroxyl group are trans if the carboxylate moieties are held cis to each other, such as would be the case in a Fischer projection of the molecule. The stereospecific synthesis of Gawron and Fondy involved the trans opening of the oxide ring of 3,4-epoxy-2,5-dimethoxytetrahydrofuran with $LiAl^2H_4$. The resulting racemic product was then hydrolyzed with acid to the corresponding malic dialdehydes. Oxidation of these dialdehydes with nitric acid gave the above racemic mixture of malic acid. The spectrum of this mixture obtained by high-resolution nuclear magnetic resonance (NMR) gave a coupling constant of 4.4 Hz for the protons at carbons 2 and 3. In contrast, the enzymatically-synthesized 2S-[3-^2H]malic acid gave a coupling constant of 6-7 Hz (Krasna, 1958; Alberty and Bender, 1959). When enzymatically-prepared 2S-[3-^2H]aspartic acid was treated with nitrous acid, the configuration at carbon 2 was retained leading to 2S-[3-^2H]malic acid. A coupling constant of 6 Hz was observed for this compound by Krasna (1958). Moreover, the transformation of the above 2S-[3-^2H] aspartic acid into 2R-[3-^2H]malic acid resulted in a coupling constant of 4 Hz.

These observations implied that aspartase and fumarase were both stereospecific for the proton erythro to the leaving group. Since the carboxylate groups of fumarate and those of 2S-aspartate in the preferred conformation were trans, then as illustrated in Figure 1, the addition and elimination reactions proceeded by a trans course. Subsequent to the determination of the steric course of the reactions catalyzed by fumarase and aspartase, a number of other enzymes have been shown to catalyze eliminations which are trans. Some of the more

FIGURE I

The trans nature of the reversible reaction catalyzed by aspartase. It is assumed that the carboxylate groups are trans to each other in the conformation of 2S-aspartate preferred at the active site. Note that the proton removed is erythro to the amino group. This becomes more obvious if carbon 2 (α carbon), shown as the forward one in the Newman projection, is rotated 180°.



2S-ASPARTATE FUMARATE

well-known enzymes include β-methylaspartase (Bright et al., 1964); histidase (Givot et al., 1969); aconitase (Englard, 1960); adenylosuccinase (Miller and Buchanan, 1962); and argininosuccinase (Hoberman et al., 1964). According to Rose (1970), trans additions or eliminations are a general feature of enzymes catalyzing reactions of this type.

Enzyme Kinetic Methodology

Before proceeding into a discussion of the various techniques by which the kinetic mechanisms of enzyme-catalyzed reactions are determined, it is in order to present a brief review of the nomenclature and certain other aspects of such mechanisms. As suggested by Cleland (1970), substrates involved in enzymatic reactions are usually designated by letters at the beginning of the alphabet (A, B, C) in the order in which they add to the enzyme. Products are identified by letters near the end of the alphabet (P, Q, R) in the order in which they are released from the enzyme. Stable enzyme forms are denoted by the letters E through G, in which E usually represents the free enzyme. In some cases, E may reflect the free enzyme complexed with a metal cofactor. Enzyme-reactant complexes in which all substrates or products are at the active site are called central complexes. Kinetic constants determined for the different reactants are written as K, K_d, and K_i and refer to the Michaelis, dissociation, and inhibition constants, respectively. V_1, v_1 and V_2, v_2 are the maximal and initial velocities, respectively, in the forward and reverse directions.

Kinetic mechanisms of enzyme reactions generally fall into one of two major categories. In the first, all reactants must be combined

with the enzyme before the chemical reaction or release of products can take place. Such a scheme is called a sequential mechanism in which the free enzyme is the only stable form. In the second category, one or more products may be released before all substrates have combined with the enzyme. Cleland (1970) describes this mechanism as being "ping-pong" in that the enzyme usually oscillates between two or more stable forms. With enzymes catalyzing reactions involving three or more substrates or products, it is not unusual for the appropriate scheme to contain both sequential and ping-pong components. A sequential mechanism is considered to be ordered if the substrates combine with the enzyme and the products are released in an obligatory fashion. If the binding of substrates or release of products is not obligatory, then the mechanism is described as being random. Finally, if certain steps in a random or an ordered mechanism appear to be essentially at thermodynamic equilibrium in the steady state, then the term rapid equilibrium is applied to the mechanism (Cleland, 1970).

In two excellent reviews, Cleland (1967, 1970) presents three principle techniques for use in studying kinetic mechanisms. These include initial velocity, inhibition, and isotope exchange studies. Fortunately, the number of plausible mechanisms for any particular enzyme is not large, and in the above reviews, Cleland shows intuitively how the resulting initial velocity and inhibition patterns can be used to eliminate various kinetic mechanisms for an enzyme. In another article, Cleland (1963) describes in more detail some of the mechanisms possible for enzyme-catalyzed reactions involving two or more substrates or products. He also gives the corresponding general rate equations as derived by the

method of King and Altman (1956). In each of the different techniques to be discussed shortly, the steady state is assumed to prevail. This is reasonable if the concentrations of the reactants greatly exceeds that of the enzyme. Also, in subsequent discussions, enzymes mechanisms involving up to two substrates or products will be used as examples.

Initial Velocity Studies. In initial velocity studies, the concentration of one substrate is usually varied at different fixed levels of the other with no products being initially present. The initial velocity for each set of conditions is determined by a suitable method. Then for each level of the non-varied substrate, a plot of 1/v versus the reciprocal of the varied substrate concentration is constructed. If the enzyme is not allosteric, a family of linear plots will result. If a sufficient number of points has been obtained, a second family of similar plots is made except that the original, non-varied substrate is now varied at different levels of the other substrate which was, of course, varied in the first series. In each case, the extrapolated ordinate intercepts are regarded as the maximal velocities that would be observed at infinite concentrations of the varied substrate in the presence of the indicated levels of the fixed substrate. If the enzyme operates by a sequential mechanism, the linear double-reciprocal plots of each family will intersect at a point to the left of the vertical axis. The general initial rate equation describing this mechanism is given as (Cleland, 1967; 1970):

$$v_1 = \frac{V_1 [A][B]}{K_{dA} K_B + K_B [A] + K_A[B] + [A][B]} \qquad (1)$$

If A is considered to be the first substrate to bind with the enzyme in an ordered mechanism, then $-1/K_{dA}$ is the horizontal coordinate of the common

intersection point in plots of 1/v versus 1/[A]. However, the corresponding coordinate when B is varied is given by $-K_A/K_{dA}K_B$. In a random mechanism,on the other hand, the coordinate of the intersection point when B is varied is $-1/K_{dB}$. Unfortunately, since the above equation is algebraically symmetrical with respect to A and B, the actual order of addition, if any, of the two substrates cannot be distinguished at this point.

The numerical values of K and V_1 are customarily determined from replots of the vertical intercepts of the primary double-reciprocal plots versus the reciprocal of the concentrations of the non-varied substrate. For example, in the replot of the appropriate intercepts versus 1/[B], the ordinate intercept is the reciprocal of V_1 at infinite concentrations of A and B. The abscissa intercept is $-1/K_B$, from which K_B, at saturating [A], can easily be calculated.

Ping-pong mechanisms, in contrast to sequential types, result in parallel primary double-reciprocal plots of the variable substrate in the presence of fixed levels of the other. The initial rate equation is similar to Equation 1 except there is no $K_{dA}K_B$ term in the denominator. The absence of this term is the reason for the observed parallel plots (Cleland, 1970).

Inhibition Studies. The second major technique for studying enzyme mechanisms is inhibition studies. Generally, an inhibitor can be viewed as any substance which combines with an enzyme or enzyme form and reduces the amount able to react with the substrate(s) present. In inhibition studies, the initial velocity is determined for various concentrations of substrate in both the absence and the presence of

different fixed levels of inhibitor. Double-reciprocal plots are then constructed in the usual manner to obtain the characteristic inhibition patterns.

Presently, there are three recognized types of inhibition patterns depending upon the way in which the inhibitor interferes with the reaction velocity (Cleland, 1970). For the sake of simplicity, the inhibitor will be considered as binding reversibly with its target enzyme form. If the inhibitor and variable substrate combine with the same enzyme form, the slopes of the double-reciprocal plots are changed; whereas, the vertical intercepts are not. This behavior is called competitive inhibition since saturation of the system with the varied substrate eliminates any inhibition, and the plots intersect on the ordinate axis. If the inhibitor and variable substrate combine with different enzyme forms that are connected by reversible steps, then noncompetitive inhibition results. Double-reciprocal plots show changes in both the slopes and vertical intercepts because saturation with the variable substrate cannot eliminate the inhibition. Lastly, if the inhibitor and substrate combine with different enzyme forms but there is an irreversible step intervening between these forms, then the double-reciprocal plots are parallel, and the inhibition is said to be uncompetitive. In inhibition studies, irreversible steps result mainly from the addition of a substrate at saturating levels or the departure of a product against a zero value of its initial concentration.

Secondary replots of slopes or intercepts of primary plots versus inhibitor concentration afford the determination of K_i as well as the complexity of the inhibition. For cases of simple inhibition, the replots are linear, and the abscissa intercept is $-K_i$. In more complex

situations, such as the combination of additional inhibitor molecules with the enzyme forms, the replot may be curved upward at increasing concentrations of inhibitor. The complexity can also be determined by the use of a series of Dixon plots (Dixon and Webb, 1964). Here the reciprocal of initial velocity is plotted against inhibitor concentration at fixed levels of substrate. As expected, linear plots suggest simple inhibition, but curved plots are obtained for the complex cases.

Some of the most useful inhibitors of an enzyme-catalyzed reaction are the products themselves. Since a product forms the same enzyme-reactant complex that it does as a substrate, inhibitions corresponding to one of the three types described previously can be expected. Therefore, when either substrate is varied in the presence of fixed levels of each product, the resulting inhibition patterns are very helpful in deducing the sequence of substrate binding and product release.

As an example, consider an ordered mechanism involving two substrates and two products. In this situation, only the first substrate binding to the enzyme will be competitively inhibited by the last product released. When the first product P is the inhibitor, noncompetitive patterns are expected with either A or B as substrates since A and B combine with enzyme forms different from that to which P combines. Also, a noncompetitive pattern is observed when B is varied in the presence of Q. If the sequence is random and the rate-limiting step of the reaction is the interconversion of E-A-B to E-P-Q (rapid-equilibrium), all product inhibition patterns are expected to be competitive. However, between those reactants that can combine with the enzyme to form inactive, dead-end complexes, the patterns are noncompetitive (Cleland, 1970). In

certain dehydrogenases, a dead-end complex may form between the NAD^+ and the oxidized reactant.

Isotope Exchange Studies. The third technique of importance to the kineticist consists of isotope exchange studies. There are two approaches to the design of exchange experiments (Cleland, 1970). In the first approach, the exchange of label between a product containing the isotope and a substrate is measured while the reaction is proceeding in the forward direction (initial velocity conditions). This is particularly useful for sequence studies of reactions involving one substrate and two or more products. The product which exchanges very slowly, with the exchange being dependent upon the presence of the other product(s), may very well be the last one released. In the second approach, isotope exchanges between labeled products and substrates are determined under conditions of chemical equilibrium. The initial velocity and product inhibition studies described previously measure only the net rate of an enzyme-catalyzed reaction. However, in isotope exchange studies, the observed rate of exchange between a pair of reactants is a direct measure of the individual flux rate of the reactants involved in the exchange. At chemical equilibrium exchanges between individual pairs of reactants are occurring even though there is no net chemical reaction. In other words, the exchange of all reactant concentrations is equal, but isotope exchanges between different reactant pairs may not necessarily be equal (Cleland, 1967).

The idea of equilibrium isotopic exchange was first proposed by Boyer (1959). Since then, the technique has been expanded by Boyer and Silverstein (1963) and Cleland (1967). Exchange studies have been

used mainly to support or contradict the results of earlier reports on the sequence of substrate binding and product release for various enzymes. For example, the data of Silverstein and Boyer (1964 a and b) have shown that for lactate dehydrogenase, the nucleotides must add before lactate or pyruvate can bind and that alcohol dehydrogenase is partly random.

Equilibrium exchange studies have also been helpful in locating the rate-limiting step of enzyme reactions (Boyer and Silverstein, 1963; Cleland, 1967). If the interconversion of the central complexes in either ordered or random sequences is rate limiting, then exchange rates of the products into their respective substrates will be essentially equal. If the rapid-equilibrium assumption for a random mechanism is not valid, i.e. release of a product is the rate-limiting step in the reaction, then the exchange rates between reactant pairs will not be equal. In the case of E. coli galactokinase, which proceeds by a random mechanism, the nucleotide exchange was 2.5 times faster than the sugar-sugar phosphate exchange (Gulbinsky and Cleland, 1968).

Kinetic Studies on Specific Ammonia-Lyases. The interactions of certain divalent metal ions, substrate and products with β-methyl-aspartase were studied in detail by initial velocity methods (Bright and Silverman, 1964; Bright, 1965). The results suggested that at pH 8.0, a complex of Mg^{2+} and threo-3-methyl-2S-aspartate, the natural substrate, probably did not function as a significant substrate. Furthermore, the divalent metal activator and substrate appeared to add randomly to the enzyme in the deamination reaction. In the reverse direction, mesaconate and NH_4^+ in the presence of constant $[K^+]$ and $[Mg^{2+}]$ bound

to the enzyme in a sequential manner. Unfortunately, product inhibition studies which would easily establish the mode of product release have not as yet been done.

The exchange of each product into threo-3-methyl-2S-aspartate during deamination was investigated in an attempt to locate the rate-determining step of the β-methylaspartase reaction (Bright, 1964). During deamination of the β-deuteriated substrate, the ^{2}H exchanged with solvent ^{1}H at a rate faster than the formation of mesaconate. This eliminated either breakage of the C-H bond or release of the abstracted hydrogen from the enzyme as the rate-limiting event. In contrast, the exchange rate of either NH_4^+ or mesaconate into substrate was small compared to mesaconate formation. Therefore, the dissociation of either product from the enzyme was not likely to be rate-limiting.

Givot et al. (1969) found urocanate to be a non-competitive inhibitor of the deamination of histidine in the histidase reaction. Although NH_4^+ was not tested, the implication could be made that urocanate was released prior to NH_4^+. As far as aspartase is concerned, the majority of the initial velocity work dealt with the pH-dependent cooperative effects of substrate and cofactors. Nevertheless, Sekijo et al. (1965) found that the deamination of 2S-aspartate was inhibited competitively by fumarate. Emery (1963) and Suzuki et al. (1973) reported that hydroxylamine also inhibited competitively the forward reaction catalyzed by both the E. coli and E. aerogenes enzymes. Yet, no inhibition studies with NH_4^+ were done. Using an incubation solution containing non-equilibrium concentrations of $N^{2}H_4^+$, fumarate, and 2S-aspartate in

^2H_2O, Englard (1958) found that following the inclusion of aspartase, ^2H was incorporated into aspartate at essentially the same rate as aspartate was produced. This observation was contrary to the rapid β-proton exchange characteristic of the β-methylaspartase reaction.

Kinetic Isotope Effects

Information gained about an enzyme's kinetic mechanism from initial velocity, product inhibition, and isotope exchange studies can generally be supplemented by the use of kinetic isotope effects. Also the chemical mechanism involved in the reaction may be deduced under certain conditions. There are two kinds of kinetic isotope effects that can result from the judicious substitution of atoms with their corresponding isotopes (Wiberg, 1964; Richards, 1970; Collins and Bowman, 1970). If the bond to the isotope is broken during the reaction, a primary isotope effect occurs. If, however, the bond in question is not broken, but undergoes a change in hydridization during the reaction, a much smaller effect results which is appropriately termed a secondary isotope effect. The major factor which contributes to the former type is the lower zero-point energy possessed by the bond to the isotope. Since more energy is required, that bond breaks more slowly than the corresponding bond in the unlabeled compound. On the other hand, a secondary isotope effect is due to the difference in the responses of the two isotopes to a change in bond hybridization, such as in going from sp^3-to-sp^2 bonding in olefin formation (Richards, 1970). The non-labeled compound can pass to the sp^2 hybridization state more easily (and thus react faster) than its labeled counterpart. In both cases, the magnitude of the effect is governed by the relative mass difference between the atom and its isotope.

The substitution of ^{2}H or ^{3}H for ^{1}H results in effects much larger than those expected from the replacement of, say, ^{14}N with ^{15}N. Note that in exchange studies, the effects which may be encountered from the use of isotopes of carbon and nitrogen are usually very small compared to the magnitudes of the differences in exchange rates.

If the isotopically substituted atom is involved in the rate-limiting step of a reaction, the accompanying isotope effect will show up as a difference in net reaction rates. Accordingly, both primary and secondary isotope effects have been used in investigations of rate-determining steps of chemical reactions. Richards (1970) states that values of primary hydrogen isotope effects ($k/k_{isotope}$) ranging from 2 to 15 can be regarded as evidence that cleavage of the carbon-hydrogen bond is rate limiting. Conversely, the absence of an effect implies that the bond scission is not rate determining. Secondary isotope effects have also been important in studying carbonium-ion mechanisms. For example, in solvolytic reactions with carbonium ions as intermediates, secondary effects (k_H/k^2_H) observed for single ^{2}H substitutions range around 1.12-1.20 (Schmidt et al., 1969).

If it can be shown by other methods that the interconversion of the central complexes is rate limiting in an enzyme-catalyzed reaction, then isotope effects can be used in conjunction with isotope exchange (especially with hydrogen) to gain a great deal of information about the chemical mechanism present. As an application, consider the aspartase-catalyzed deamination of 2S-aspartate at pH 7.0. For general reviews of the various mechanisms of elimination reactions, refer to Ingraham (1962); Bordwell (1972); Saunders and Cockerill (1973). Apparently, β-eliminations can proceed by a spectrum of mechanisms, but for the sake of simplicity, the

discussion will be confined to the three major types that are possible for the deamination as illustrated in Figure 2.

In Mechanism A, the hydrogen and the amino group are lost in a single, concerted step to give fumarate. In B, the proton is abstracted first, giving an intermediate carbanion which eliminates ammonia in a subsequent step forming fumarate. Lastly, in C, C-N bond fission may occur first producing an intermediate with carbonium-ion character. This intermediate loses the proton in going to fumarate. Furthermore, either one of the two steps in Mechanism B or C could be rate limiting.

Table II presents the expected isotope effects when 2S-aspartate, labeled with ^{2}H at the α or β carbons or with ^{15}N at the α carbon, is the substrate. Also included for each mechanism is the possibility of β-proton exchange occurring at a rate faster than fumarate production when 2S-aspartate is deaminated in the presence of $^{2}H_2O$. At first glance, it appears as if one would not be able to distinguish between a carbanion mechanism in which C-H bond breakage was rate limiting or the carbonium-ion case in which the second step was slow. Fortunately, Ingraham (1962) is of the opinion that those eliminations with slow ionizations of hydrogen may be uncommon.

As will be described in detail later, the labeled forms of 2S-aspartate given in Table II were synthesized by enzymatic methods and applied to the study of the aspartase reaction. To this end, it was necessary to determine not only the degree of isotope enrichment[2] but also if ^{2}H had been incorp-

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[2]Throughout this dissertation the use of "isotope enrichment" is to be interpreted as the total amount of the label at the location under consideration and, therefore, includes the natural abundance of the label.

FIGURE 2

Possible chemical mechanisms of the deamination of 2S-aspartate catalyzed by aspartase at pH 7.0. For purposes of clarity, no catalytic groups on the enzyme that may be involved in the reaction have been included.

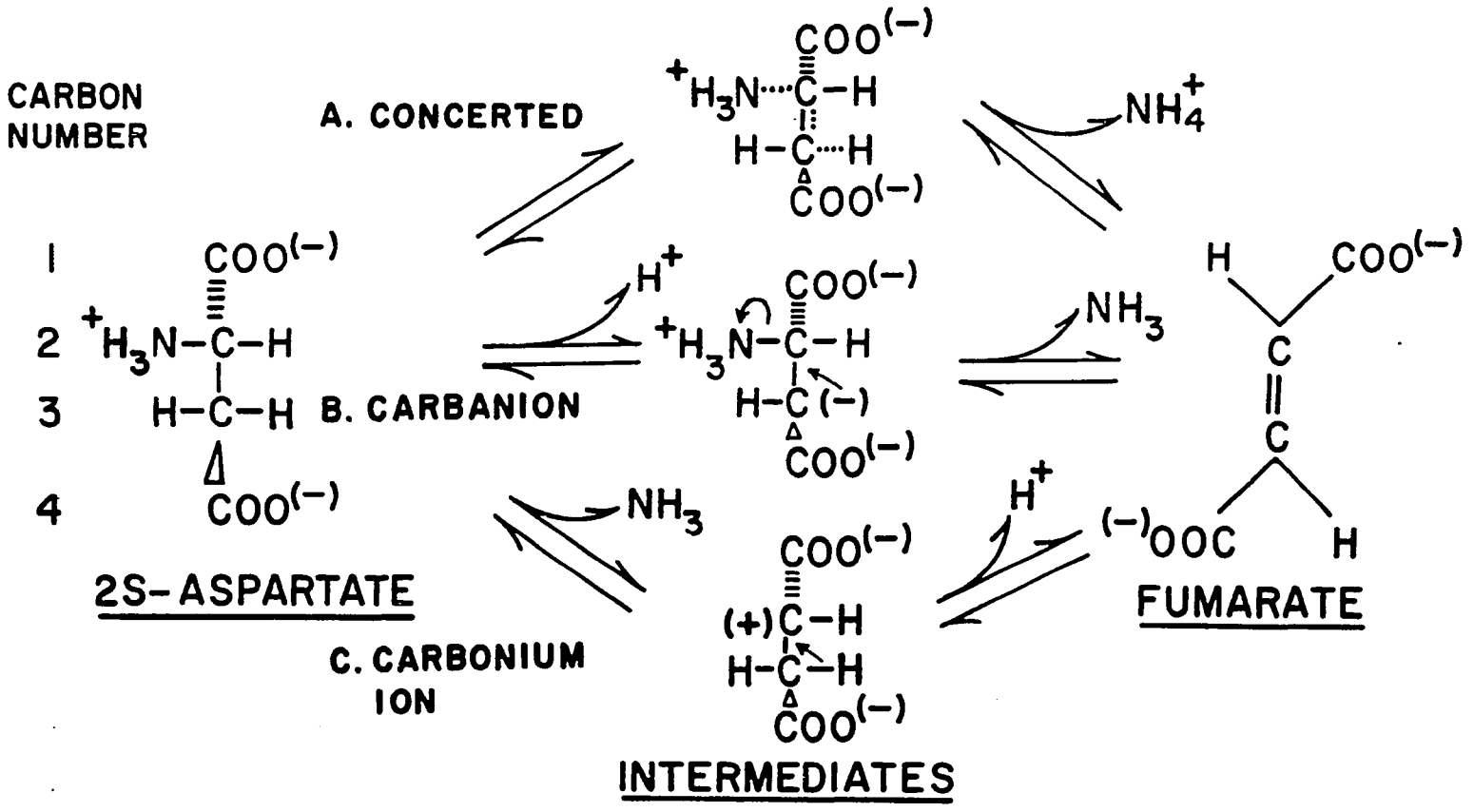

CARBON NUMBER
1
2
3
4
A. CONCERTED
B. CARBANION
C. CARBONIUM ION
2S-ASPARTATE
FUMARATE
INTERMEDIATES
NH4+
NH3
H+

TABLE II

Kinetic Primary and Secondary Isotope Effects Expected to be Observed for Each of the Chemical Mechanisms Suggested for the Aspartase Reaction in Figure 2. Also included for each mechanism is the possibility of exchange of ^{2}H from $^{2}H_2O$ into the β position of 2S-aspartate that is faster than fumarate formation during the initial forward reaction.

		SUBSTRATE AND TYPE OF ISOTOPE EFFECT			
Mechanism	Slow step of Reaction	2S,3R-[3-^{2}H] Aspartate (Primary)	2S-[2-^{15}N][a] Aspartate (Primary)	2S-[2-^{2}H] Aspartate (Secondary)	Occurrence of β Exchange
Concerted	Both	Yes	Yes	Yes	No
Carbanion	First	Yes	No	No	No
	Second	No[b]	Yes	Yes	Yes
Carbonium ion	First	No	Yes	Yes	No
	Second	Yes	No	No	No

[a]The apparent primary isotope observed for the ^{15}N-labeled aspartate would be small due to the small relative mass difference between ^{14}N and ^{15}N.

[b]A small primary isotope may be observed (Bada and Miller, 1970) which should be substantially smaller than the case in which the first step is rate limiting.

orated at other carbon positions during the syntheses of the ^{2}H-labeled species. For example, in the preparation of 2S-[2-^{2}H]aspartic acid, advantage was taken of the known α-proton exchange between substrate and solvent catalyzed by glutamate-oxaloacetate transaminase (Tamiya and Oshima, 1962; Kobayashi and Makino, 1969). However, Whalen and Long (1969) reported that the same enzyme catalyzed the exchange of both β protons as well as the α proton of 2S-glutamate.

The aspartase reaction was used to synthesize the other labeled forms of 2S-aspartic acid. Although aspartase itself was shown to be stereospecific (Englard, 1958; Krasna, 1958), α proton exchange could have occurred during the synthesis of 2S,3R-[3-^{2}H]aspartate if the enzyme preparation had been contaminated with an appropriate transaminase. It was therefore imperative to discover if significant labeling had occurred at the β carbon of 2S-[2-^{2}H]aspartic acid and at the α position of the 2S,3R-[3-^{2}H]aspartic acid. Fortunately, this problem was handled adequately by the techniques of NMR and mass spectroscopy.

Bada and Miller (1970) applied certain of these isotope methods to a study of the non-enzymatic, reversible deamination of 2RS-aspartate. In the pH range from 5.8 to 12.4, the rate of exchange of the β protons of aspartate with ^{2}H was shown by NMR to exceed that of fumarate formation. Moreover, these workers found only a small isotope effect for the deamination of enzymatically prepared 2S,3R-[3-^{2}H]aspartate. These observations are suggestive of a carbanion mechanism in which the rate-limiting step is C-N bond breakage. A similar mechanism has been proposed for the deamination of threo-3-methyl-2S-aspartate catalyzed by β-methylaspartase (Bright, 1964). It also is based on the observation of a rapid β-proton exchange compared to product formation and no β-^{2}H primary isotope

effect.

Although in an enzyme-catalyzed reaction, isotope effects are observed whenever the rate-limiting step involving the isotopes is the interconversion of the central complexes, the converse may not be true, especially with regard to secondary effects. An effect obtained experimentally with labeled substrates does not necessarily mean that the chemical events of the reaction are rate limiting. If some subsequent step is rate determining, then the labeled and unlabeled species of the substrate could yield different equilibria. Since the equilibrium constant enters into the general steady-state rate equation for the reaction (Hanson and Havir, 1972), any discrimination against the labeled species would show up as a difference in rates (Rose, 1970). As a result, erroneous conclusions concerning the chemical mechanism could be made from a consideration of isotope-effect data alone.

A classic example of this is seen in the dehydration of 2S-malate catalyzed by fumarase. Alberty et al. (1957) found no isotope rate effect during the dehydration of 2S,3R-[3-^{2}H] malate nor any evidence of β-proton exchange that was faster than could be accounted for by the hydration of fumarate. In addition, a secondary ^{2}H effect was observed in the initial reaction of 2S-[2-^{2}H]malate (Schmidt et al. 1969). These data suggested a carbonium-ion mechanism in which breaking of the C-O bond occurred as the slow step. Yet, when the reaction was studied by isotope exchange methods, both during the initial dehydration of malate and under conditions of chemical equilibrium, surprising results were obtained (Hansen et al. 1969). The hydroxyl group exchanged more rapidly from the solvent into unreacted malate than was incorporated by the back reaction.

Furthermore, in the equilibrium studies, the oxygen exchange was 4 and 2.5 times faster than the hydrogen and carbon exchanges, respectively. The slow exchange between the ^{2}H of substrate and the ^{1}H of water was not due to an isotope effect, for the rates of exchanges with ^{2}H and ^{3}H were found to be the same. It follows, then, that the rate-limiting step was actually product release, and the observed secondary isotope effect was likely an equilibrium effect.

In the next three chapters, the four primary tools of the kineticist, namely initial velocity, product inhibition, isotope effect, and isotope exchange studies, have been applied to an investigation of the mechanism of action of aspartase. A kinetic mechanism has been proposed and various aspects of it have been discussed. Finally, a decision has been made regarding the validity of using isotope effects as direct probes of the organic mechanism of the aspartase reaction.

II. EXPERIMENTAL PROCEDURES

Reagents and Materials

2S-aspartic acid was obtained from K and K Laboratories or Mann Research Laboratories. Fumaric acid was a product of Eastman Organic Chemicals or J. T. Baker Chemical Co. These acids were recrystallized from hot water, dried, and converted to their potassium salts, pH 7.0, for use in the various kinetic studies. 2S-[^{3}H]aspartic acid (Generally Labeled; 2.15 Ci/mmole) and [1,4-^{14}C]fumaric acid (57 mCi/mmole) were purchased from New England Nuclear Corp. and Amersham/Searle Corp., respectively. These compounds were recrystallized a suitable number of times with their corresponding unlabeled forms prior to use.

The $^{15}NH_4Cl$ from Bio-Rad Laboratories had an enrichment of 99 atom % ^{15}N; whereas, that from Stohler Isotope Chemicals contained 95 atom % ^{15}N. Deuterium oxide with enrichments of 99.8 atom % ^{2}H and 99.86 atom % ^{2}H were obtained from International Chemical and Nuclear Corp. and Bio-Rad Laboratories, respectively. Amersham/Searle Corp. was the source of tritiated water (200 mCi/ml).

Glutamate-oxaloacetate transaminase (GOT) with bound pyridoxal phosphate and with specific activities of 2×10^4 and 1×10^5 units/mg of protein was purchased from Worthington Biochemical Corp.

Sephadex G-200 and DEAE-Sephadex A-50 (capacity 3.5 $\pm$ 0.5 mequiv/g) were products of Pharmacia Fine Chemicals, Inc. Hydroxylapatite was either obtained as a powder from Bio-Rad Laboratories or prepared by the method of Tiselius (1956), except that potassium phosphate buffers were

used in the final steps instead of sodium phosphate buffers. Dowex 50W-X8 (H^+ form) and Dowex 1-X8 or X10 (Cl^- form) were of reagent grade from J. T. Baker Chemical Co.

The MgO from Matheson, Coleman, and Bell Manufacturing Chemists which was to be used in one of the distillation procedures was heated in an electric muffle furnace for 2-3 hr at 600°. The activated powder was stored in a desiccator.

All additional chemicals were of reagent grade or, when applicable, of suitable grade for enzyme studies.

Conditioning of Dowex Resins

The ion-exchange resins required for the purification of the different forms of labeled aspartic acid usually contained impurities which had to be removed before the resins could be used. The procedures described below for cleaning and conditioning Dowex 50W and Dowex 1 were taken in part from Moore and Stein (1951).

A sufficient quantity of Dowex 50W-X8 (20-50 mesh) was placed in a large fritted funnel, and enough 4 N HCl was added to cover the resin. After the resin was gently agitated for a brief period of time, the HCl solution was allowed to drip through the funnel at a reasonably slow rate. The addition of more HCl with gentle stirring and subsequent filtering was repeated until the filtrate was colorless. Usually, no more than 10 volumes of 4 N HCl was necessary. The resin was washed free of acid with deionized water and treated with enough 1 N NaOH to make the filtrate alkaline. The resin was transferred to a large beaker containing about 3 volumes of 1 N NaOH. The suspension was gently stirred for 3 hr in a

hot water bath (90-98°). The supernatant was conveniently removed by aspiration. This cycle of adding 1 N NaOH and heating was repeated until the wash became clear, at which time, the resin was converted to the H^+ form with 1 N HCl.

After it was washed free of acid in the next step, the resin was treated with 3 to 6 volumes of 95% ethanol which was removed by once again washing the resin with deionized water. It was subsequently washed with 3 to 4 volumes of 1 N NaOH, and the filtrate was scanned from 220 to 340 nm in a Beckman DB spectrophotometer. If any absorbing materials were present, then the above treatments with 1 N HCl, ethanol, and 1 N NaOH were repeated. Otherwise, the conditioned resin was converted to the H^+ form and degassed for 2 hr before pouring the column.

The strategy for conditioning the Dowex 1-X10 resin (200-400 mesh) was similar. However, the resin was washed with 95% ethanol only when it was in the Cl^- form. During treatment with base, at least 20 volumes of 1 N NaOH were required and heating was avoided. Subsequent to washing the resin with deionized water, it was converted back to the Cl^- form, washed with more water, and the filtrate scanned for absorbing material as before. Any absorbance between 220-280 nm was reduced by storing the resin in ethanol overnight, washing with water, and repeating the treatment with 1 N NaOH. The resin was converted to the acetate form by equilibrating with 4 N potassium acetate until the wash was Cl^- free.

Preparation of Aspartase

Aspartase was prepared from sonic lysates of Enterobacter aerogenes, subspecies alvei (formerly Bacterium cadaveris; ATCC-9760). The cells were grown in three, 10-liter batches of medium containing 1% yeast extract,

1% tryptone, and 0.5% K_2HPO_4. Growth from a 20% inoculum was allowed to proceed for 48 hr at 30° without aeration or agitation. The inoculum cultures were previously grown with aeration for 25 hr at 30°. The K_2HPO_4 solutions were always autoclaved separately from the other components contained in the carboys. In this manner, formation of precipitates during the required 90-min sterilization period was avoided.

Throughout its purification, aspartase was routinely assayed by the method of Williams and Lartigue (1967). The reaction mixture contained in a total volume of 3.0 ml: 50 mM Tris-HCl buffer, pH 7.0; 1 mM $MgSO_4$; 0.1 mM EDTA; and 50 mM potassium 2S-aspartate, pH 7.0. Upon addition of 0.1 ml of the enzyme solution the initial velocity (v_1) of the reaction was determined by the change in absorbance at 240 nm in either a Beckman Model DB (29°) or a Gilford Model 2000 recording spectrophotometer (28°). As the light path through the cuvette was 1 cm in length, an extinction coefficient of 2.53 ml/μmole (Emery, 1963) was used to convert values of ΔA_{240}/min to μmoles of fumarate/min per ml of assay solution. Since the preparations obtained in the initial steps of the purification scheme contained relatively large amounts of inert protein, the enzyme was usually added to both the reference and sample cells. The substrate, of course, was omitted from the reference cell. In the later steps, the aspartase preparations were assayed for fumarase activity. This assay solution contained in a total volume of 3.0 ml: 50 mM Tris-HCl buffer, pH 7.0; 1 mM $MgSO_4$; 0.1 mM EDTA; and 50 mM potassium 2S-malate, pH 7.0.

Formerly, protein concentrations were determined by the methods of Lowry et al. (1951) or Exton (1925). However, the use of a biuret procedure was found to be more convenient. The biuret reagent was made accord-

ing to Layne (1957). Standards of bovine serum albumin contained phosphate or Tris-HCl buffers at approximately the same concentrations as in the enzyme solutions being assayed. Some runs were made in which the reagent blank consisted of 0.05 M phosphate buffer, pH 7.0, Triton-urea, and biuret. Other runs utilized both a reagent blank identical with the one above, but with 3% NaOH instead of the biuret reagent, and a blank containing 3% NaOH, Triton-urea, and protein to serve as a blank for the enzyme solution.

Although the present purification procedure is somewhat similar to that of Williams and Lartigue (1967) at least in the initial steps, it will be described here in its entirety, both as a convenience to the reader and because some modifications in the early steps have been made.

Step 1: Sonication. After the cells were thawed, approximately 50 g were suspended in 3 volumes of cold 0.1 M potassium phosphate buffer, pH 7.0, containing 1 mM mercaptoethanol. The suspension was subjected to sonication with a Branson Model LS-75 sonifier for 5 min at maximum amperage. An alcohol-ice bath was used during the sonication. The lysate was centrifuged in the Beckman L-2 ultracentrifuge at 105,000g (Roto No. 30) for 1 hr at 0 to 2°. All potassium phosphate buffers used in subsequent steps were also 1 mM in mercaptoethanol. The temperature was maintained at or below 4° throughout the remainder of the procedure.

Step 2: Protamine Sulfate Precipitation. The solid material from the above centrifugation was discarded, and the protein concentration of the clear amber supernatant was determined. If necessary, this solution was diluted with 0.1 M phosphate buffer, pH 7.0, until the protein concentration was about 30 mg/ml. A quantity of protamine sulfate equal to 17%

of the total protein in the supernatant was dissolved in a sufficient volume of the same buffer to give a 1% solution. This entire solution was then added dropwise to the supernatant over a period of 15 to 30 min with continuous stirring. After an additional 15 min of stirring, the suspension was centrifuged at 27,000g for 30 min, and the precipitate was discarded.

Step 3: pH Fractionation. The retained supernatant was brought to pH 4.55 by the rapid, dropwise addition of cold 2 M acetic acid. The resulting suspension was immediately centrifuged at 27,000g for 30 min. The thick, gummy precipitate was resuspended in a solution of 0.05 M phosphate buffer, pH 7.0, containing 10 μM $MgSO_4$ with the aid of several short sonications at 3 amps. The volume used was one-third of that of the supernatant remaining after precipitation with protamine sulfate. The suspension was centrifuged at 27,000g for 20 min, and the precipitate was again discarded.

Step 4: Ammonium Sulfate Fractionation. Following the addition of a 0.05 volume of 1 M phosphate buffer, pH 7.4, to the above supernatant it was brought to 35% saturation with $(NH_4)_2SO_4$. After being stirred for 15 min, the suspension was centrifuged at 27,000g for 30 min, and the precipitate was discarded. The supernatant was brought to 55% saturation with $(NH_4)_2SO_4$, stirred for 15 min, and centrifuged to collect the precipitate. At this point, the precipitate could be frozen with little loss of activity.

Step 5: Sephadex G-200 Chromatography. The frozen $(NH_4)_2SO_4$ precipitate was dissolved in 3 to 5 ml of 0.05 M phosphate buffer, pH 7.0. The solution was carefully applied to a column, 2.5 x 36 cm, of Sephadex G-200

which had been previously equilibrated with 0.05 M phosphate buffer. Elution was achieved with the same buffer solution using a flow rate of 10 ml/hr. Normally, 3- to 4-ml fractions were collected. In this and in the chromatographic steps to follow, the eluent was monitored at 280 nm by an ISCO Type 6 optical unit attached to an ISCO Model UA-5 absorbance monitor.

Step 6: Hydroxyapatite Chromatography. Those fractions from the Sephadex G-200 step containing aspartase activity were combined and placed on a column, 1.2 x 11 cm, of hydroxyapatite that had been previously equilibrated with 0.05 M phosphate buffer, pH 7.0. The column was eluted successively at pH 7.0 with 25-ml volumes of 0.1, 0.2, and 0.3 M phosphate buffer solutions and overnight with a 0.5 M phosphate buffer solution. The flow rate remained approximately 8.0 ml/hr. Fractions were assayed for the presence of fumarase as well as for aspartase. The recovered aspartase was dialized overnight in 0.05 M phosphate buffer, pH 7.0.

Step 7: DEAE-Sephadex A-50 Chromatography. Prior to its use, a suitable amount of DEAE-Sephadex A-50 was swollen and activated in the presence of saturated KCl. A boiling water bath was used to decrease the length of time required for this process. After the resin had been washed free of Cl^-, it was equilibrated with 0.05 M Tris-HCl buffer, pH 7.0, and degassed before pouring the column. After a column, 2.5 x 29 cm, was prepared, it was washed overnight with 0.05 M Tris-HCl buffer, pH 7.0, made 1 mM in mercaptoethanol.

The dialyzed enzyme solution was carefully loaded on the DEAE-Sephadex column via a syringe. The enzyme was eluted with 0.05 M Tris-HCl buffer,

pH 7.0, containing 1 mM mercaptoethanol and a gradient from 0.0 to 0.3 M KCl. The total volume of the gradient solution was 300 ml, and the enzyme appeared near the end. Those fractions with the highest aspartase activity were combined and used in the various kinetic studies. With some preparations, elution of the enzyme from the column was achieved by successive washings with appropriate volumes of 0.05 M phosphate buffer solutions, pH 7.0, containing 0.0, 0.15, 0.25, and 0.3 M KCl.

Synthesis of 2S-Aspartic Acid Stereospecifically Labeled with ^{2}H, ^{3}H, or ^{15}N

2S,3R-[3-^{2}H]Aspartic Acid and 2S-[2-^{15}N]Aspartic Acid. Aspartic acid stereospecifically labeled with ^{2}H at the 3R position or ^{15}N at the α carbon were kindly synthesized by the late Dr. Virginia R. Williams. Essentially, the procedure for both included incubation at pH 7.0 of aspartase with fumarate as the sodium or potassium salt and either NH_4Cl in $^{2}H_2O$ or $^{15}NH_4Cl$ in H_2O. The labeled aspartates were recovered and purified as the acids by ion-exchange chromatography and recrystallization.

2S-[2-^{2}H] Aspartic Acid. 2S-[2-^{2}H]aspartic acid was prepared by a variation of the procedure of Tamiya and Oshima (1962). In a typical preparation, 1 g of 2S aspartic acid was dissolved in approximately 35 ml of deionized water with the addition of 5 N KOH to obtain the potassium salt. The pH was adjusted to 7.6 with 0.1 N KOH. After 5 ml of 0.1 M potassium phosphate buffer, pH 7.6, was added, the solution was lyophilized to dryness. The glassy residue was dissolved in approximately 25 ml of $^{2}H_2O$, and the lyophilization was repeated. To insure an essentially complete replacement of exchangeable protons with ^{2}H, the residue was once again dissolved in $^{2}H_2O$ and lyophilized. Finally, the residue

was redissolved in ^2H_2O and brought to a final volume of 50 ml. GOT (5 mg; original specific activity: 1 x 10^5 units/mg) and a drop of toluene were added to the solution.

After the solution was incubated at room temperature for 25 hr, the enzyme was denatured by heating the solution to 95-100° for 10 min. Activated charcoal (100 mg) was added, and the suspension was filtered. The collected charcoal was washed once with hot dionized water.

The procedure for isolating the labeled aspartate was taken largely from Zintel et al. (1969). The above filtrate was applied to a column, 2.5 x 94 cm, of Dowex 50W-X8 (H^+ form). The column was washed with 1.5-2.0 l of deionized water from a reservoir to which a few drops of toluene had been added. The flow rate was maintained at 120 ml/hr. The amino acid was eluted with a solution of 1 M ammonium acetate-acetic acid, pH 6.5. Aliquots from the fractions collected were applied to a sheet of Whatman No. 3 paper which was subjected to rapid electrophoresis at pH 1.9 and 4 Kvolts for 15 min in a Savant LT48A tank electrophorator. The dried paper was developed with ninhydrin in acetone. The fractions containing aspartate were combined and lyophilized. The residue was dissolved in 30 ml of water, the pH was adjusted to 7.0 with NH_4OH, and the solution was applied to a column, 2.5 x 94 cm, of Dowex 1-X10 (acetate form). Subsequent to washing the column with 1.5 l of deionized water, the aspartate was eluted with 1 N acetic acid. The amino acid was recovered by lyophilization and recrystallized three times from water and ethanol. Similar yields were obtained when Dowex 50W-X10 and Dowex 1-X8 were substituted as the ion-exchangers.

2S-[2-^{3}H]Aspartic Acid. 2S-[2-^{3}H]aspartic acid was synthesized and purified by either of two methods. In the first procedure, the reaction solution contained in a total volume of 20 ml: 1 mM potassium phosphate buffer, pH 7.5; 50 mM potassium 2S-aspartate; 3 mg of GOT (6×10^4 units); and 500 mCi of tritiated water. After the solution was incubated for 4 hr at room temperature, it was brought to a pH of 3.0 with HCl and placed in ice overnight to effect crystallization of the labeled 2S-aspartic acid. Further precipitation of acid occurred upon the addition of acetone. The suspension was filtered, and the precipitate was dissolved in water with the aid of a minimum amount of a KOH solution. The solution was applied to a column of Dowex 1-X8 (acetate form) which was then washed for 2 days with water to remove exchangeable ^{3}H from the aspartate. Elution was achieved with 1 N acetic acid. The combined fractions containing the amino acid were subjected to flash evaporation to remove the acetic acid. When the volume was sufficiently reduced, water was added and subsequently removed for four cycles. Crystallization of aspartic acid was induced by the addition of acetone.

The second procedure was found to be more convenient and was similar to that used in the synthesis of the 2S-[2-^{2}H] aspartic acid. In this case, the reaction mixture contained in a final volume of 25 ml: 0.01 M potassium phosphate buffer, pH 7.6; 0.15 M potassium 2S-aspartate; 1 Ci of tritiated water; 2.5 mg (2.5×10^5 units) of GOT; and a drop of toluene. Following a 12-hr period of incubation at room temperature, the pH was lowered to 1 with 3 N HCl thereby denaturing the enzyme.

After the mixture was lyophilized twice the residue was dissolved in approximately 8 ml of water. The labeled aspartic acid was purified by passage first through a column of Dowex 50W-X10 (H^+ form) and then through

one of Dowex 1-X8 (acetate form). The recovered product was recrystallized from ethanol and water. The crystals were stored in the freezer in a desiccator. The specific radioactivity of tritiated aspartic acid prepared by the second method was 0.323 mCi/mmole.

Analysis of 2S-Aspartic Acid for ^2H enrichment by Nuclear Magnetic Resonance Spectroscopy.

The degree of ^2H enrichment at the stereoselective positions of the labeled forms of 2S-aspartic acid was determined by NMR. Information about the absence of ^2H at other non-labile locations was also implied.

To this end, 0.5 mmole each of the α- and β-deuterated species and the unlabeled acid was dissolved in 1-2 ml of 1 N HCl and lyophilized. The residues were redissolved in ^2H_2O and lyophilized two additional times. They were each carefully dissolved in 0.75 ml of 3 N [^2H]trifluoroacetic acid and transferred by filtration through cotton plugs into corresponding NMR tubes just prior to analysis. The spectra were obtained with the Perkin Elmer R12B. This instrument was unable to detect concentrations of aspartic acid in the same solvent system which were less than 2% of those of the labeled amino acids. In other words, if at least 98% of the α protons had been replaced by ^2H, then no signal due to any remaining α protons would have been detected.

Since the areas of the signals for the α and β protons were directly related to their abundances, the extent of ^2H incorporation at carbon 3 of 2S,3R-[3-^2H]aspartic acid was estimated from the expression:

$$\text{Atom \% } ^2H = (2 - \frac{\beta}{\alpha}) \times 100 \qquad (2)$$

in which the β/α term was the ratio of the area of the β-proton signal to that of the α-proton signal. A ratio of unity would imply a β enrichment of ≥ 98 atom % ^{2}H.

Any significant incorporation of ^{2}H at the β carbon of 2S-[2-^{2}H]aspartic acid was obvious from a comparison of its β-proton signal with the corresponding signal of the unlabeled aspartic acid. The ^{2}H enrichment at the α position of 2S,3R,-[3-^{2}H]aspartic acid was determined by a similar comparison of the α proton signals of this and the unlabeled acid.

Analysis of 2S-Aspartic Acid for ^{2}H or ^{15}N Enrichment by Mass Spectroscopy

Fragmentation Procedure. To check the reliability of the quantitative NMR technique, the ^{2}H enrichments of the two ^{2}H-labeled forms of 2S-aspartic acid were also obtained by electron-impact mass spectroscopy. Moreover, the ^{15}N content of 2S-[2-^{15}N]aspartic acid was estimated in this manner. The approach involved converting each labeled acid into a suitably volatile derivative which, in turn, was introduced into a Hitachi-Perkin-Elmer RMS-4 mass spectrometer. The isotopic content was calculated from the intensities of selected peaks in the resulting cracking pattern. Furthermore, the presence or absence of label at other positions in the molecule was suggested by the characteristics of the pattern.

Biemann et al. (1961) reported the fragmentation patterns of the free ethyl ester derivatives of a number of amino acids, including the diethyl ester of 2S-aspartic acid. In fact, Biemann and Deffner (1961) successfully determined the ^{15}N enrichment in mixtures of ^{15}N-labeled amino-acid ethyl esters without their prior separation into individual components.

They employed a stepwise volatilization scheme to introduce the esters into the mass spectrometer. Hochrieter and Schellenberg (1969) obtained a satisfactory cracking pattern with the dimethyl ester hydrochloride of glutamic acid, implying that isolating the derivative as the free ester was not necessary. According to Biemann et al. (1961), however, the methyl esters of α-amino acids tended to dimerize into diketopiperazines more readily than their ethyl ester counterparts. As a consequence, the diethyl ester hydrochlorides of the labeled forms of aspartic acid appeared to be the derivatives of choice. The following description was typical for preparing the derivatives.

A round bottom flask containing 50 ml of absolute ethanol was chilled in an ice bath. To the ethanol was added 11 ml of acetyl chloride dropwise from a pipet while the contents of the flask were being stirred. When the evolution of heat had ceased, the flask was sealed with a ground-glass stopper, and stirring was continued at ambient temperature for 5 hr. At the end of this time, approximately 80 mg of labeled 2S-aspartic acid was added, and the reaction solution was stirred overnight.

The following day, the solvent was removed under reduced pressure and 25°. More ethanol was added and its evaporation repeated. The residue was treated once with ethyl acetate, and the white precipitate that formed was dissolved in a minimum volume of absolute ethanol and transferred to a break-seal ampule. The contents were concentrated by flushing with nitrogen under reduced pressure.

At this point, a slurry of silica gel (60-200 mesh) in diethyl ether was poured into a glass column 1.5 cm in diameter. After a sufficient settling time, the ether and any gel was removed to give a column 30 cm

in height. The concentrated reaction solution was carefully layered on the column. The diester hydrochloride of the amino acid was eluted first from the column with a solvent system composed of benzene and absolute ethanol (75:25, v/v). The flow rate was maintained at about 30 drops per min, and 5- to 10-ml fractions were collected. To assay for the presence of diester or starting materials, each fraction was concentrated under reduced pressure. A few μl of the remaining volume was applied to a microscope slide coated with a thin layer of silica gel. When 2 or 3 samples had been applied, the slide was developed in the same benzene-absolute ethanol solvent used with the column. After drying, the slides were sprayed with ninhydrin and acetone to locate the compounds.

Those fractions containing the diester hydrochloride were combined and the volume concentrated as before. The residue was treated with ethyl acetate, dissolved in small volume of absolute ethanol, and transferred with filtering into a second break-seal ampule. The residue remaining after evaporating the ethanol was washed with ether which was carefully removed by suction. Following solution of the white precipitate in a minimum volume of ethanol, sufficient diethyl ether was added to induce crystallization in the freezer.

The crystals were recovered by filtration and dried at room temperature before they were sealed in a clean ampule. The melting point found for the diethyl ester hydrochloride of 2S-aspartic acid was 107°-109°. Greenstein and Winitz (1961) reported a value of 109°-110° for the same compound.

The mass-spectral cracking pattern was obtained for the diethyl ester

hydrochloride of each labeled aspartic acid. The isotope enrichments were estimated with the following equation described by VandenHeuval et al. (1970):

$$A_e = \frac{\Delta I \, A_o^2}{N + \Delta I \, A_o} + A_u \qquad (3)$$

In the expression, A_e was the enrichment in the heavier isotope of the pair; A_u was the natural abundance of the heavier isotope; A_o was the natural abundance of the lighter isotope, and N was the number of locations in the molecule at which the heavier isotope was incorporated. The ΔI term was the difference between the intensities found for the heavier-isotope peak in the spectrum of the enriched compound and the corresponding peak in that of the unlabeled compound. After the intensities of the two lighter isotope peaks were set to unity, those of the two heavier isotope peaks were adjusted accordingly, and the resulting difference was taken as ΔI. The values for A were necessarily expressed as decimals. Therefore, $A_e \times 100$ gave the enrichment in atom %.

Residual Gas Analysis. Unlike the isotope content of the ^2H-labeled acids, that of 2S-[2-^{15}N]aspartic acid was determined by a second mass-spectroscopic technique which was also based on electron impact. In this method, the labeled amino acid was degraded by a Kjeldahl procedure (Bremner, 1965b), and the nitrogen was recovered as $(^{15}NH_4)_2SO_4$. The $[^{15}N]NH_4^+$ was oxidized to $[^{15}N]N_2$ gas with NaOBr. The enrichment found was compared with the corresponding value obtained from the cracking pattern of the diethyl ester derivative. In this way it was hoped that the reliability of the latter procedure would be reinforced. The procedure for the residual-gas analytical technique is now described

in more detail.

Aliquots of 2 ml from a 55 mM solution of sodium 2S-[2-^{15}N]aspartate were transferred to steam distillation flasks. To remove any NH_3 that may have initially contaminated the labeled amino acid, 1 ml of 0.5 N NaOH was added, and the flasks were immersed in a boiling water bath for 10 min. To each solution, when it had sufficiently cooled, was added 2 ml of concentrated H_2SO_4 and 0.5 g of a powdered mixture consisting of 10 parts K_2SO_4 and 1 part $CuSO_4 \cdot 5H_2O$ (w/w). The flasks were heated in a sand bath, and several glass beads were included to reduce bumping. When the solutions had cleared, the digestion was continued for an hour.

Each flask was again cooled before diluting the contents with 10 ml of water and attaching it to the all-glass steam distillation apparatus illustrated in Figure 3. Immediately, 11 ml of 10 N NaOH was added from the funnel, and steam distillation was commenced. The distillate was collected in 100-ml flasks containing 3 ml of 0.1 N H_2SO_4 at a rate between 7 and 8 ml per min. At the end of 4.5 min, the distillation was terminated; the tip of the condenser and the neck of the receiving flask were rinsed. The volume of the distillate was then reduced by boiling with the inclusion of glass beads to prevent bumping. Between samples, the apparatus was steamed with deionized water for several min. A flask with 2 ml of concentrated H_2SO_4 only was treated in an identical manner as the digested samples. This was considered as a blank.

When the volume in each receiving flask was reduced to approximately 2 ml, the contents were transferred to a screw-capped, graduated centrifuge

tube. Rinses were likewise included until no more than 3.5 ml was present in the tube. The contents of the flask serving as a blank were transferred as quantitatively as possible and diluted to 8 ml. The concentration of NH_4^+ present in this solution was determined by Nesslerization.

For each mass-spectral analysis, a 3-ml aliquot was treated with the NaOBr reagent. Any CO_2 that may have formed was removed by passing the evolved N_2 through a U-tube immersed in liquid nitrogen before its introduction into the ionization chamber of a Dupont Model 21-614 residual gas analyzer. The heights of the peaks at m/e 28, 29, and 30 were obtained. Subsequently, the background was subtracted from each signal, and the net values were used in Equation 3 (Bremner, 1965c) to calculate the enrichment of the $[^{15}N]N_2$.

$$\text{Atom \% } ^{15}N = \frac{h_{29} + 2h_{30}}{2(h_{28} + h_{29} + h_{30})} \times 100 \qquad (4)$$

In this equation, h_{28}, h_{29}, and h_{30} were the net peak heights observed at m/e 28, 29, and 30, respectively. Since all of the steps from the digestion of the labeled aspartic acid to the production of $[^{15}N]N_2$ were essentially quantitative, then isotope effects were probably of no significance, and the enrichment found in the $[^{15}N]N_2$ was identical to that of the original amino acid. A correction was made, if necessary, for the isotope dilution that might have occurred as a result of the presence of any NH_4^+ in the concentrated H_2SO_4 used in the digestion step.

Kinetic Procedures

Initial Velocity, Product Inhibition, and Isotope Effect Studies.

In these kinetic studies, unless otherwise indicated, the typical assay

mixture contained in a total volume of 3.0 ml: 50 mM Tris-HCl buffer, pH 7.0; 1 mM $MgSO_4$; 0.1 mM EDTA; and appropriate concentrations of substrate(s) and, when tested, of product. Magnesium ion was present at a concentration approximately 60 times its K_m as determined for aspartase from Escherichia freundii by Sekijo et al.(1965). Stock solutions of substrates or products were freshly prepared prior to each study. The initial velocity was determined by a change in absorbance at 240 nm in an appropriate recording spectrophotometer. In the product inhibition studies of the deamination reaction, K^+ concentration was maintained at 50 mM.

As 2S-aspartic acid could be prepared with nearly complete ^2H or ^{15}N incorporation at stereoselective locations in the molecule, kinetic isotope effects were determined from a comparison of the initial deamination rates of 2S,3R-[3-^2H]aspartate, 2S-[2-^2H]aspartate, and 2S-[2-^{15}N]aspartate with those of the unlabeled 2S-aspartate. An isotope effect was considered to exist if the rates (v_{2_H}, v_{15_N}) of the corresponding labeled species were significantly less than those (v_{1_H}, v_{14_N}) of the unlabeled form. The concentrations of both ^2H-labeled substrates were varied over a predetermined range. In the ^{15}N isotope effect experiments, however, the rates for only one concentration of the substrates were compared. In this instance each reaction solution contained in a final volume of 3.1 ml: 19.4 mM potassium phosphate buffer, pH 6.9; 9.7 mM substrate as the potassium salt; 1.7 mM $MgSO_4$; 90 μM EDTA; and 0.1 ml of enzyme. A large number of initial rates was determined for each solution.

The 2S-[2-^3H]aspartic acid prepared earlier was used in a different approach to verify the results found with the ^2H species. The reaction

system was designed to contain in a final volume of 20 ml: 50 mM potassium phosphate buffer, pH 7.0; 13.1 mM potassium 2S-[2-^{3}H]aspartate, 1.5 mM $MgSO_4$; 0.15 mM EDTA; and 2 ml of aspartase (4 units). The above solution was initially made to 18 ml. Before enzyme was added, each of two, 1.8 ml aliquots was discharged into 0.1 ml of 3 N HCl, followed by 0.2 ml of enzyme. These were to serve as zero-time controls. To the remaining reaction mixture, 1.6 ml of enzyme were added, and 2-ml aliquots were quenched with 0.1 ml of 3 N HCl after selected intervals of time. A secondary isotope would be apparent if the specific radioactivity of the produced fumarate changed with time.

Isotope Exchange Studies. In a preliminary experiment, the exchange of ^{2}H with the 3R proton of 2S-aspartate during enzymatic deamination was followed by NMR. The buffered reaction system in ^2H_2O was contained in the NMR tube itself, with the added enzyme having been dialyzed earlier in [^{2}H] phosphate buffer. In spite of the appearance of fumarate, the ratio of the signals due to the α and β protons of the aspartate indicated that no exchange of ^{2}H into the remaining substrate had occurred. This approach suffered in that to obtain measurable integration patterns, a large excess of 2S-aspartate relative to enzyme had to be used. As a consequence, the high background of substrate could have prevented the observation of an exchange which was due to the amination reaction or was faster than the formation of fumarate.

Fortunately, the exchange reaction could be studied with a more sensitive method involving ^{3}H. To 2.0 ml of the standard aspartase assay solution, 0.5 ml of tritiated water (specific activity 200 mCi/ml) was added, and the solution was allowed sufficient time to equilibrate ^{3}H.

There was no need to add immediately the enzyme since it had been shown earlier that no non-labile ^{3}H was incorporated into 2S-aspartate in the absence of enzyme. After the preparation of a zero-time control, 0.1 ml of enzyme (130 μg protein) was included, and 0.1 ml aliquots were quenched with 10 μl of 3 N HCl at selected times during the reaction. An identical reaction, but without tritiated water, was performed concurrently to monitor the production of fumarate. The fumarate concentrations in the quenched samples of this series were determined at 240 nm from a standard curve obtained under the same conditions.

The equilibrium exchange rates of carbon and nitrogen isotopes from [1,4-^{14}C]fumarate and [^{15}N]NH_4^+ respectively, into 2S-aspartate were measured at two sets of reactant concentrations. The first incubation solution contained 122.3 mM 2S-aspartate, 25.0 mM [^{15}N]NH_4^+ (28.7 atom % ^{15}N), and 25.0 mM [1,4-^{14}C]fumarate. One milliliter of this mixture gave 2.22 x 10^5 cpm. The second solution contained 36.0 mM 2S-aspartate, 61.3 mM [^{15}N]NH_4^+ (28.7 atom % ^{15}N), and 3.0 mM labeled fumarate which gave 2.72 x 10^4 cpm/ml of reaction mixture. In both cases, 10-ml samples contained 50 mM Tris-HCl, pH 7.0; 1 mM $MgSO_4$; 0.1 mM EDTA; 1 mM mercaptoethanol, and 1 ml of aspartase (300 μg).

Initially, each incubation solution was made to 90 ml and allowed to reach 28° prior to the addition of any enzyme. The zero-time control consisted of 9 ml of this solution and 1 ml each of 6 N H_2SO_4 and enzyme. To the remainder of the incubation mixture, 9 ml of the aspartase preparation was added to initiate the isotope exchanges. Ten-milliliter aliquots were transferred as quickly as possible to plastic, screw-capped tubes which were incubating at 28°. The reactions in the tubes were

quenched with 1 ml of 6 N H_2SO_4 after specific intervals of time and immediately frozen until the analyses could be done. The contents of the last tube was incubated for approximately 24 hr before the enzyme was denatured. The relative concentrations of fumarate in the samples were determined to insure that equilibrium conditions had been maintained throughout the experiment.

Separation of 2S,3R-[3-^{3}H]Aspartate from 2S,3R-[3-^{3}H]Malate and Assay for ^{3}H.

The enzyme preparation used in the ^{3}H-exchange study of aspartate deamination was found to contain fumarase. Its presence, however, was not a hindrance to the interpretation of the exchange data. All that was required was the separation of the aspartate from the labeled malate before assaying for radioactivity. This was accomplished by the ascending chromatographic procedure described below.

A chromatogram with a convenient number of marked lanes, each 1.25 inches wide, was prepared from Whatman No. 3 paper. At the origin of each lane, 10 µl of the corresponding quenched sample was applied. After allowing the samples to dry for a sufficient length of time, the chromatogram was developed in an ascending tank with a solvent system composed of n-butanol, acetic acid, and water (60:15:25 by volume). When the solvent front had traveled between 9.5 and 10 inches, the chromatogram was removed and dried.

For the radioactivity determinations, the chromatogram was cut into strips along the edges of the lanes. A plastic leader strip, preferably of the same width, was attached to each end of the first paper strip.

These leaders were wound around the feed and uptake reels of a Packard Model 7201 radiochromatogram scanner to keep the paper strip properly aligned and taut when it was fed between the Geiger detectors. The zero-time control strip was scanned to insure that the level of labile ^{3}H in 2S-aspartic acid had been reduced to background counting levels. The location of aspartic acid was visualized by dipping this strip into a ninhydrin-acetone solution. Each of the other strips was positioned along the side of the control strip, and the area on the sample strip corresponding to the aspartic acid spot was marked. The strips were then scanned in succession. No more than two peaks of radioactivity were observed away from the origin at any one time. The one nearest possessed the same R_f as the aspartic acid on the control strip; whereas, the one furthest away was conclusively identified as 2S-malic acid.

The level of radioactivity represented by a particular peak was directly proportional to the net area under that peak after correcting for background. The total area and the background to be subtracted from it were easily obtained with the help of the mechanical integrator which was part of the Honeywell Electronik 18 chart recorder. The net area was subsequently converted into cpm per peak. Moreover, it was desirable to place the observed appearance of ^{3}H into aspartate and malate on the same basis as the production of fumarate. Therefore, to transform the data expressed in cpm per peak into μmoles per ml, the efficiency of the detectors had to be determined.

For this purpose, the recrystallized 2S-[^{3}H]aspartic acid (Generally Labeled) was dissolved in 0.05 N HCl, and the solution was diluted to 25 ml with the same acid. A specific radioactivity of 2.796 μCi/ml for

this solution was determined by liquid scintillometry in which [^{3}H]toluene was the internal, primary standard. To each of three lanes of a chromatogram of Whatman No. 3 paper, a 10-µl aliquot of the secondary standard was applied. After the chromatogram was developed with the same solvent system as before, the strips were dried and subsequently assayed for radioactivity. The efficiency of the detectors for counting ^{3}H under these conditions was 0.67± 0.05%.

Separation of Labeled Fumarate from Labeled 2S-Aspartate and Assay for ^{3}H and ^{14}C.

In the secondary isotope effect study involving 2S-[2-^{3}H]aspartate and in the equilibrium isotope exchange studies, labeled fumaric acid was successfully removed from the correspondingly labeled 2S-aspartic acid by extracting aliquots of the acidified reaction solutions with diethyl ether. A similar procedure was used previously by Hansen et al. (1969) to separate fumaric acid from malic acid in investigations with fumarase. It was apparent that under acidic conditions, aspartic acid would have an even greater tendency than malic acid to remain in the aqueous phase during extraction with ether.

Each quenched sample collected in the secondary isotope investigation was extracted twice with 2-ml volumes of diethyl ether. The ether phases were transferred to a small beaker and evaporated. The residue was dissolved in a suitable volume of absolute ethanol, and duplicate 0.2 ml aliquots were assayed for ^{3}H by liquid scintillometry. Also, the concentration of fumaric acid in the ethanol extract was measured at 240 nm. The specific activity of the extracted fumaric acid was reported as cpm per µmole.

In the equilibrium exchange studies, ^{14}C was initially present solely in fumarate. However, after the attainment of isotopic equilibrium, assuming an insignificant isotope effect due to ^{14}C, aspartate contained $\geq$ 5 fold more label than did the fumarate. Therefore, the aspartic acid fractions were routinely assayed for ^{14}C because, not only was greater precision attainable, but contamination due to the presence of small amounts of fumaric acid was minimized.

The extractions were carried out in 15-ml graduated centrifuge tubes with teflon-lined caps. Duplicate, 0.5-ml aliquots of the quenched reaction mixtures were extracted repeatedly with 10-ml volumes of diethyl ether saturated with 0.55 N H_2SO_4 or HCl. The ether was dispensed with a manually operated automatic pipet (Labindustries' Repipet). Tubes with ether were shaken vigorously, each for 1 min, by holding them in a horizontal position against the side of the rubber agitator of a Vortex mixer. During the period of agitation, the tubes were intermittently shaken manually to insure complete mixing of the phases. The extractions on any given tube were repeated 6 to 8 times, depending upon the concentration of fumaric acid. The ether layers were carefully transferred by gentle aspiration into a flask containing dilute base to trap the fumaric acid as the salt. Another series of tubes containing 0.5-ml volumes of the quenched samples were prepared but were not extracted. These were used to determine the total radioactivity present in each sample.

Aquasol scintillation cocktail (New England Nuclear Corp.) was dispensed in 8-ml volumes into all tubes. After the tubes were shaken vigorously to obtain clear solutions, the contents were carefully transferred to scintillation vials and assayed for ^{14}C in a Beckman Model LS-II

liquid scintillation system. Fortunately, quench corrections were unnecessary as quenching was equivalent in each vial.

The precision of the activity determinations was better than 2.0%. At least 99.8% of the fumaric acid was removed by the extraction procedure. Nevertheless, because of the high initial activity of the fumaric acid, the presence of even small amounts of fumaric acid in the extracted, early-time samples would contribute significantly to the observed activity. However, a correction for this was easily made with the following set of simultaneous equations:

$$\begin{aligned} X + Y &= T \\ X + fY &= U \end{aligned} \qquad (5)$$

In these equations, X and Y were the cpm due to aspartic and fumaric acids respectively; T was the total cpm in each time sample, and U was the cpm observed for the corresponding aqueous phases after extraction. Finally, f was the fraction of fumaric acid remaining after extraction as determined from the zero-time control.

Paper strips from a chromatogram of the 24-hr sample were scanned with the Packard Model 7201. The absence of 2S-[1,4-^{14}C]malate implied that the aspartase preparation had been fumarase free.

Separation of [^{15}N]NH_4^+ from 2S-[2-^{15}N]Aspartate and Assay for ^{15}N.

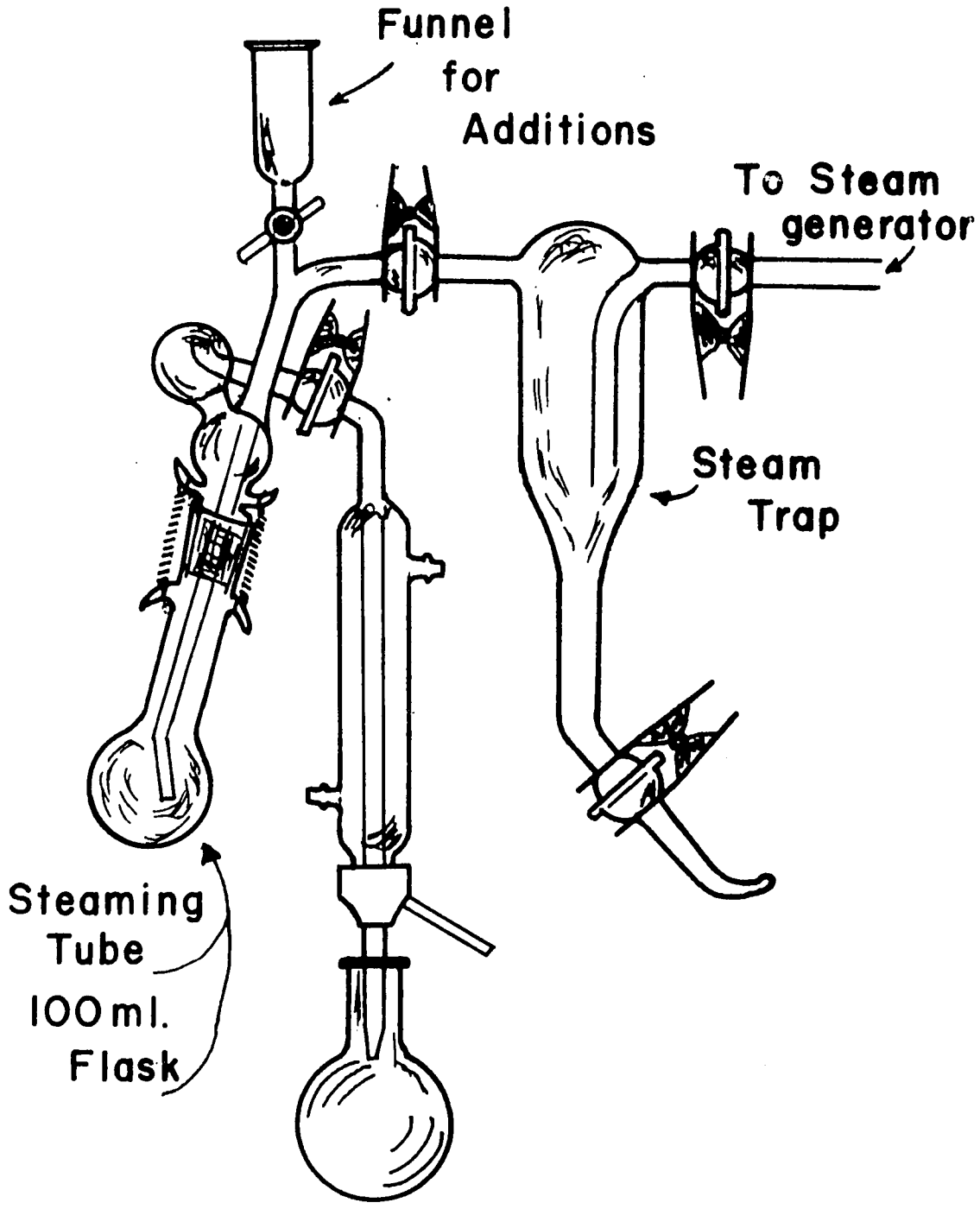
It was more convenient to determine the ^{15}N content of the [^{15}N]NH_4^+ than that of the 2S-[2-^{15}N]aspartate. But this required the quantitative removal of [^{15}N]NH_4^+ from the other nitrogenous compounds present in the

incubation mixtures, namely Tris, aspartate, and EDTA. Any ion-exchange technique was out of the question for several reasons. First, it would have been difficult to keep cation resins free of NH_4^+ contamination. Second, the anion resins that were considered contained substituted amines as an integral part of their structure. Fragments containing these amines could have easily contaminated the labeled NH_4^+ during its isolation, thereby preventing an accurate analysis for ^{15}N content. Finally, ion-exchange techniques probably would have been too time consuming. Alternative methods utilizing diffusion or distillation seemed more promising.

To obtain reliable ^{15}N measurements, at least 1 mg of labeled N_2 was needed which, of course, demanded the use of rather large sample sizes. As a consequence, Conway microdiffusion techniques were impractical. On the other hand, steam distillation methods were well suited to handle the problem. The only obstacle that had to be overcome was finding a reagent that would impart just enough alkalinity to the medium to cause the removal of the NH_4^+ (as NH_3) without affecting the other compounds during distillation. Bremner (1965a) found that during short periods of distillation not exceeding about 4 min, the inclusion of MgO in the solutions resulted in little or no breakdown of amino acids and other nitrogenous compounds present, yet, effectively removed any NH_3 contained therein. Similar results were obtained when powdered MgO was used with the apparatus illustrated in Figure 3 to distill NH_3 from solutions containing all of the compounds that were to be present in the equilibrium exchange incubation mixtures. By following essentially the same procedure

FIGURE 3

All-glass steam distillation apparatus (after Bremner, 1965a) for the quantitative separation of $[^{15}N]NH_4^+$ from either Kjeldahl digests or time samples collected in the equilibrium exchange studies. Conditions for distillation are given in the text.


Funnel
for
Additions
To Steam
generator
Steam
Trap
Steaming
Tube
100 ml.
Flask

as outlined by Bremner (1965a), recoveries of NH_3 from these test solutions as $(NH_4)_2SO_4$ was better than 98%. Ammonium concentration was always determined by the Nessler's technique.

At the beginning of a series of runs, the apparatus was allowed to steam distill for 10-15 min to clear it of any extraneous NH_3. The rate was maintained at 7-8 ml per min, and the temperature of the coolant flowing through the condenser was adjusted so that the distillate was cool to the touch.

The procedure being described was typical for the removal of $[^{15}N]NH_4^+$ from the other compounds in the quenched samples collected during the exchange studies. Depending upon the NH_4^+ concentration, duplicate aliquots of 2 or 4 ml from each sample were neutralized directly in the 100-ml distillation flasks prior to distillation. The final volumes were between 10 and 20 ml. To the neutralized contents of the first flask, 0.4 g of activated MgO was added quickly; the flask was immediately attached to the apparatus, and distillation was commenced. The distillate was collected in a 100-ml boiling flask containing 3 ml of 0.1 N H_2SO_4. The tip of the condenser was kept just above the level of the liquid. At the end of 3 min, the distillation was terminated, and after the distillation flask was removed, the steaming tube was rinsed with deionized water to remove any MgO emulsion which tended to adhere. Another flask containing deionized water was attached and distillation was continued for 1 min. The tip of the condenser and the neck of the receiving flask were rinsed with deionized water, and the volume of the contents in the flask was reduced as before. When the volume had

reached about 2 ml, the contents were transferred to screw-capped centrifuge tubes. Washings were included until a volume of 3.5 ml was reached.

Before the contents of the next flask were distilled, the apparatus was steamed for several minutes. However, between different time samples, 95% ethanol was distilled, followed by water, to remove traces of labeled NH_3 held over from the previous distillation. Blanks containing only Tris-HCl, pH 7.0, or neutralized sulfate were also carried through the entire procedure.

The $[^{15}N]NH_4^+$ in a 3-ml aligout from each tube was oxidized to $[^{15}N]N_2$ gas with NaOBr and analyzed in the Dupont residual gas analyzer. Two or three scans of the m/e range from 27 to 32 were made in succession before the remaining $[^{15}N]N_2$ was evacuated from the instrument. The ^{15}N enrichment was determined from Equation 4 as previously described. The quality of the data that was obtained for a typical time sample is illustrated in Table III. The Tris-HCl and sulfate blanks made insignificant contributions to the intensities of the m/e peaks used in the

Table III. Precision in the Determination of the ^{15}N Content of $[^{15}N]NH_4^+$ as $[^{15}N]N_2$

2-hr Sample; Scan No.	^{15}N Enrichment (Atom % ^{15}N)	
	Aliquot A	Aliquot B
1	21.36	21.40
2	21.37	21.39
Average	21.365	21.395

calculations.

Treatment of Data

Typical double-reciprocal plots of initial velocity versus substrate concentration have been shown to be hyperbolic (curved downward) at high substrate concentrations (Williams and Lartigue, 1967). Nevertheless, as discussed later, the likely cause of this hyperbolicity should not affect conclusions drawn concerning the kinetic scheme of aspartase by restricting the analysis to the linear portions of such plots. Consequently, data from the linear regions of double-reciprocal plots were often fitted to linear equations by a regression program.

The slopes from the product inhibition patterns were replotted against the product concentrations to determine the inhibition constants. Alternately, values for K_i were calculated from the intersection of Dixon plots of $1/v_1$ versus [Product]. The intercepts derived from the initial velocity patterns of the amination reaction were replotted against the reciprocal of the nonvaried substrates concentrations to find the Michaelis constants for fumarate and NH_4^+. When applicable, some kinetic constants were estimated directly from the appropriate primary plots.

The experimental data from the equilibrium exchange studies were successfully fitted to exponential equations by a nonlinear regression program. For the exchange of ^{15}N from labeled NH_4^+ into 2S-aspartate, the equation used was:

$$Y_i = (a - b)\, e^{-kt_i} + b \qquad (6)$$

in which Y_i was the experimentally observed atom % ^{15}N at time = t_i

(in hr); a and b were the enrichments at t = 0 and t = ∞ (equilibrium), respectively, and k was the rate constant for the curve. Since the appearance of ^{14}C into aspartate from fumarate was measured, the corrected values in cpm were fitted to the following expression:

$$Y_i = b(1 - e^{-kti}) \tag{7}$$

in which Y_i, b, and k had similar definitions as in Equation 6.

Equations 6 and 7 were easily converted to the expression given by Hansen et al. (1969) for estimating isotope exchange rates at chemical equilibrium. If the exchange was between reactants A and P, the appropriate form of their equation was:

$$R = - \frac{[A][P]}{[A] + [P]} \times \frac{\ln (1 - F_i)}{t_i} \tag{8}$$

In Equation 8, R was the rate of isotope interchange between A and P; [A] and [P] were the concentrations involved, and F_i was the fraction of isotopic equilibrium achieved at time = t_i. It followed that the rates of exchange of ^{15}N and ^{14}C in the aspartase reaction could be found by multiplying the appropriate concentration factors of Equation 8 by the rate constants given by Equations 6 and 7. The rates for each isotope at individual times during the course of the exchange were also estimated with Equation 8. Since Equations 6 and 7 allowed the prediction of isotope distributions at isotopic equilibrium, the experimental data could be converted into values of F_i for use in the equation. Hopefully, for the isotope under consideration, identical rates would be found at all values of F_i.

III. RESULTS

Purification of Aspartase

The best aspartase preparations obtained from E. aerogenes by the procedure described in Chapter II usually had a specific activity of approximately 60 μmoles of fumarate formed/min per mg of protein. Similar values were reported for the aspartase from E. coli by Rudolph and Fromm (1971) and Suzuki et al. (1973). In the preparations from E. aerogenes, separation of fumarase from the aspartase was achieved for the most part, as a result of chromatography on hydroxylapatite. In Figure 4, the activities associated with fumarase and aspartase were superimposed on the absorbance at 280 nm due to the presence of protein in the eluent from the hydroxylapatite column. Apparently, fumarase eluted before the aspartase. Further chromatography on DEAE-Sephadex A-50 revealed the presence of at least four protein components besides aspartase. Although the preparations were unstable, they were used in the various kinetic studies without undue complications. Nevertheless, it may be desirable at some later date to conduct experiments to determine the conditions whereby the enzyme can be stabilized.

Initial Velocity and Product Inhibition Studies

Initial Velocity Studies of the Amination Reaction. The initial velocity pattern obtained for varying the NH_4^+ concentration in the presence of fixed levels of fumarate is shown in Figure 5. The data from this pattern were replotted in Figure 6 to illustrate the initial velocity

FIGURE 4

Separation of fumarase from aspartase by chromatography on hydroxylapatite. Protein was expressed as absorbance at 280 nm. The activities associated with aspartase and fumarase were determined with an assay mixture which contained in a final volume of 3 ml: 50 mM Tris-HCl, pH 7.0; 1 mM $MgSO_4$; 0.1 mM EDTA; 50 mM substrate, and 0.1 ml of enzyme preparation. Initial rates were determined by a change in absorbance at 240 nm and 28-29°.

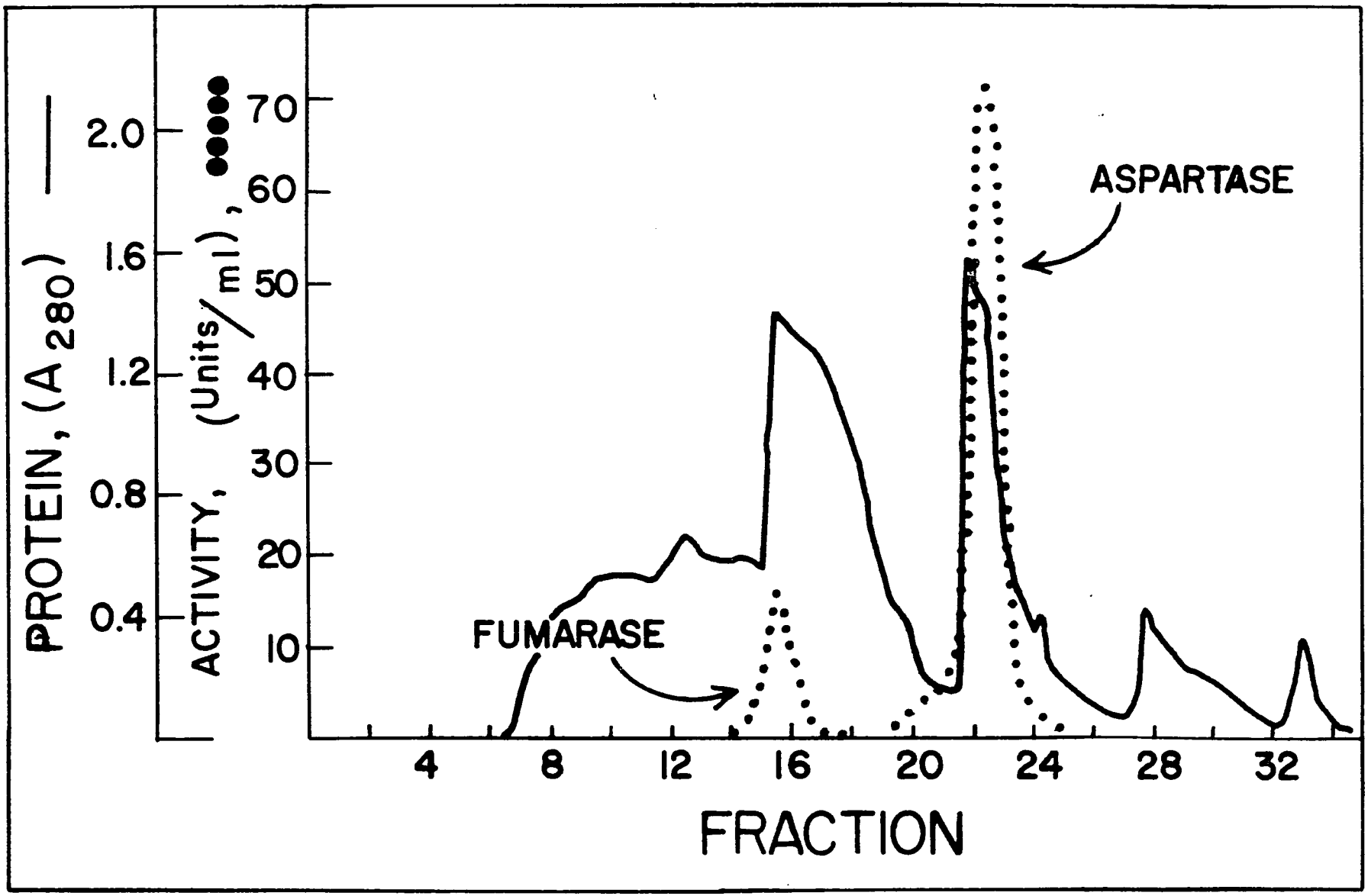

PROTEIN, (A_{280})
ACTIVITY, (Units/ml), ●●●●
2.0
1.6
1.2
0.8
0.4
70
60
50
40
30
20
10
ASPARTASE
FUMARASE
4
8
12
16
20
24
28
32
FRACTION

FIGURE 5

Double-reciprocal plots of the initial rate of fumarate amination as a function of NH_4^+ concentration at the indicated concentrations of fumarate. In addition to the substrates, each reaction solution contained Tris-HCl, pH 7.0; 1 mM $MgSO_4$; 0.1 mM EDTA, and 0.3 units of aspartase. Initial velocities were determined by a change in absorbance at 240 nm and 29° in a Beckman Model DB recording spectrophotometer. The curve for infinite fumarate concentration is a replot of the extrapolated intercepts from the pattern in Figure 6.

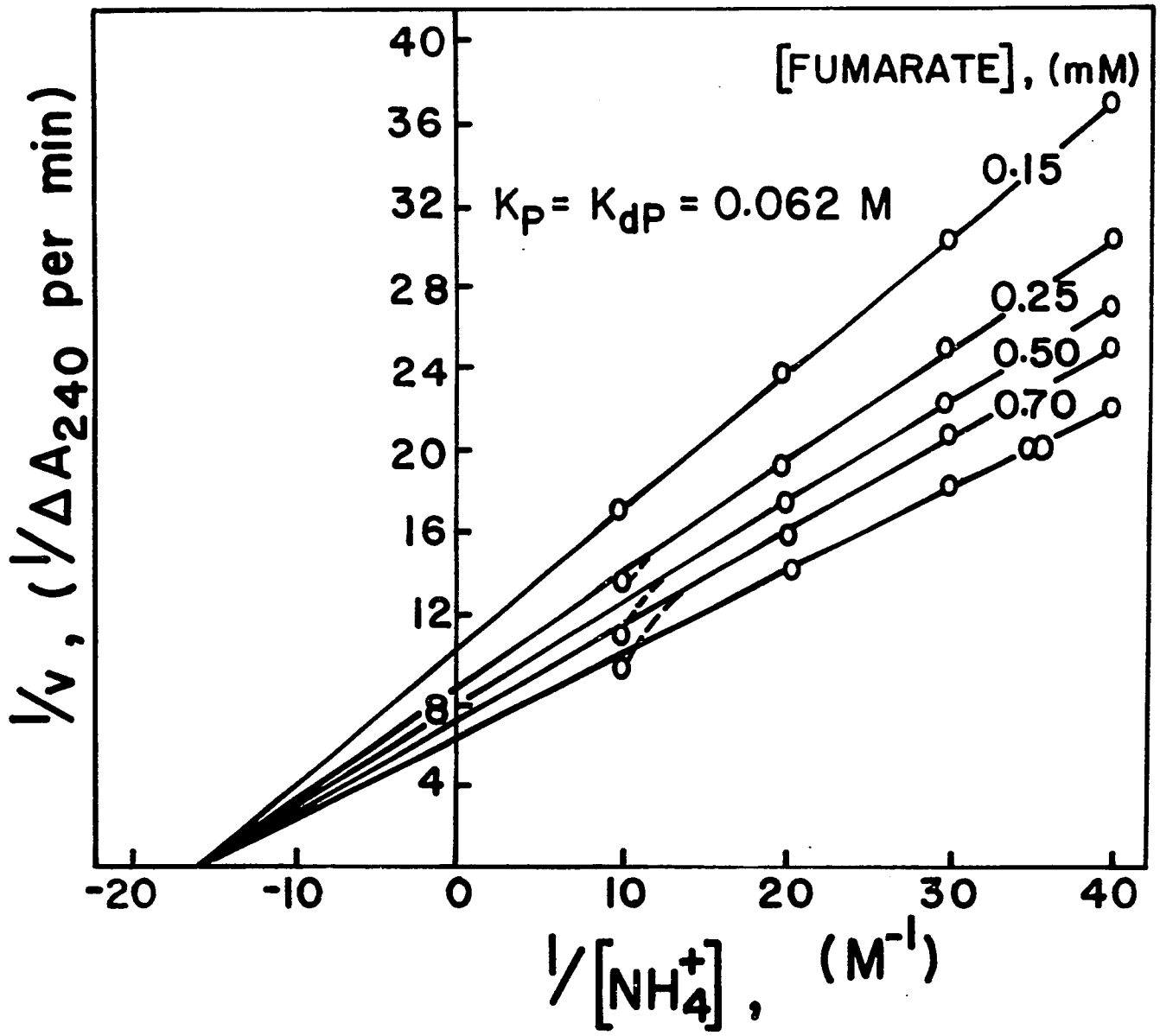
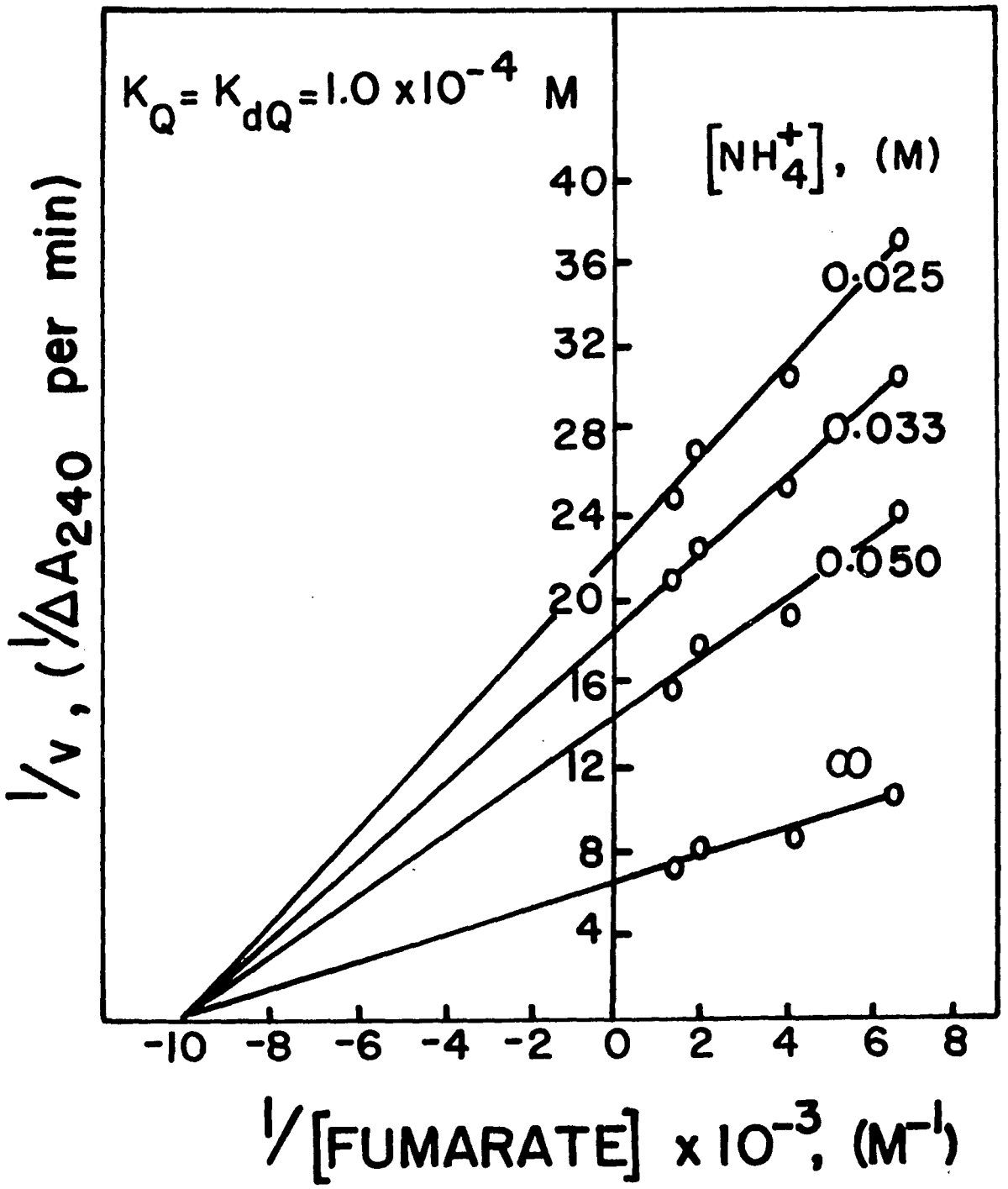

[FUMARATE], (mM)
0.15
0.25
0.50
0.70
∞
$K_P = K_{dP} = 0.062$ M
$1/v$, ($1/\Delta A_{240}$ per min)
$1/[NH_4^+]$, (M^{-1})
40
36
32
28
24
20
16
12
8
4
-20
-10
0
10
20
30
40

FIGURE 6

Double-reciprocal plots of the initial rate of fumarate amination as a function of fumarate concentration at the indicated concentrations of NH_4^+. The curve for infinite NH_4^+ concentration is a replot of the extrapolated intercepts from the pattern in Figure 5.


$K_Q = K_{dQ} = 1.0 \times 10^{-4}$ M
$[NH_4^+]$, (M)
0.025
0.033
0.050
∞
40
36
32
28
24
20
16
12
8
4
-10 -8 -6 -4 -2 0 2 4 6 8
1/v, (1/ΔA240 per min)
1/[FUMARATE] x 10⁻³, (M⁻¹)

pattern for varying the fumarate concentration in the presence of fixed levels of NH_4^+. In addition, the vertical intercepts of Figure 6, which represented the apparent maximal velocities at infinite fumarate concentration, were replotted against the reciprocal of NH_4^+ concentration in Figure 5 (∞ line). Likewise, the ordinate intercepts of Figure 5 were replotted versus the reciprocal of fumarate concentration in Figure 6 (∞ line). In each case, the extrapolated linear portions of the primary plots intersected with the intercept replot on the abscissa to the left of the ordinate axis. This suggested that the apparent K_d and K_m were equivalent for each substrate.

The data appeared to fit Equation 9 for a sequential mechanism in which $K_P \approx K_{dP}$ and $K_Q \approx K_{dQ}$.

$$\frac{V_2}{v_2} = \left(\frac{K_P}{[P]} + 1\right) \times \left(\frac{K_Q}{[Q]} + 1\right) \qquad (9)$$

In this equation, [P] and [Q] are the concentrations of P and Q, respectively. Table IV presents a comparison between the experimentally observed values for $1/v_2$ and those calculated from Equation 9 at the indicated concentrations of NH_4^+ and fumarate. At 100 mM NH_4^+, the experimental values deviated from linearity; the deviation was greater as the fumarate concentration was increased.

Product Inhibition Studies of the Deamination Reaction. As illustrated in Figure 7, NH_4^+ was found to inhibit competitively the deamination of 2S-aspartate. The linearity of the slope replot given in the inset of Figure 7 suggested that the inhibition was linear within the

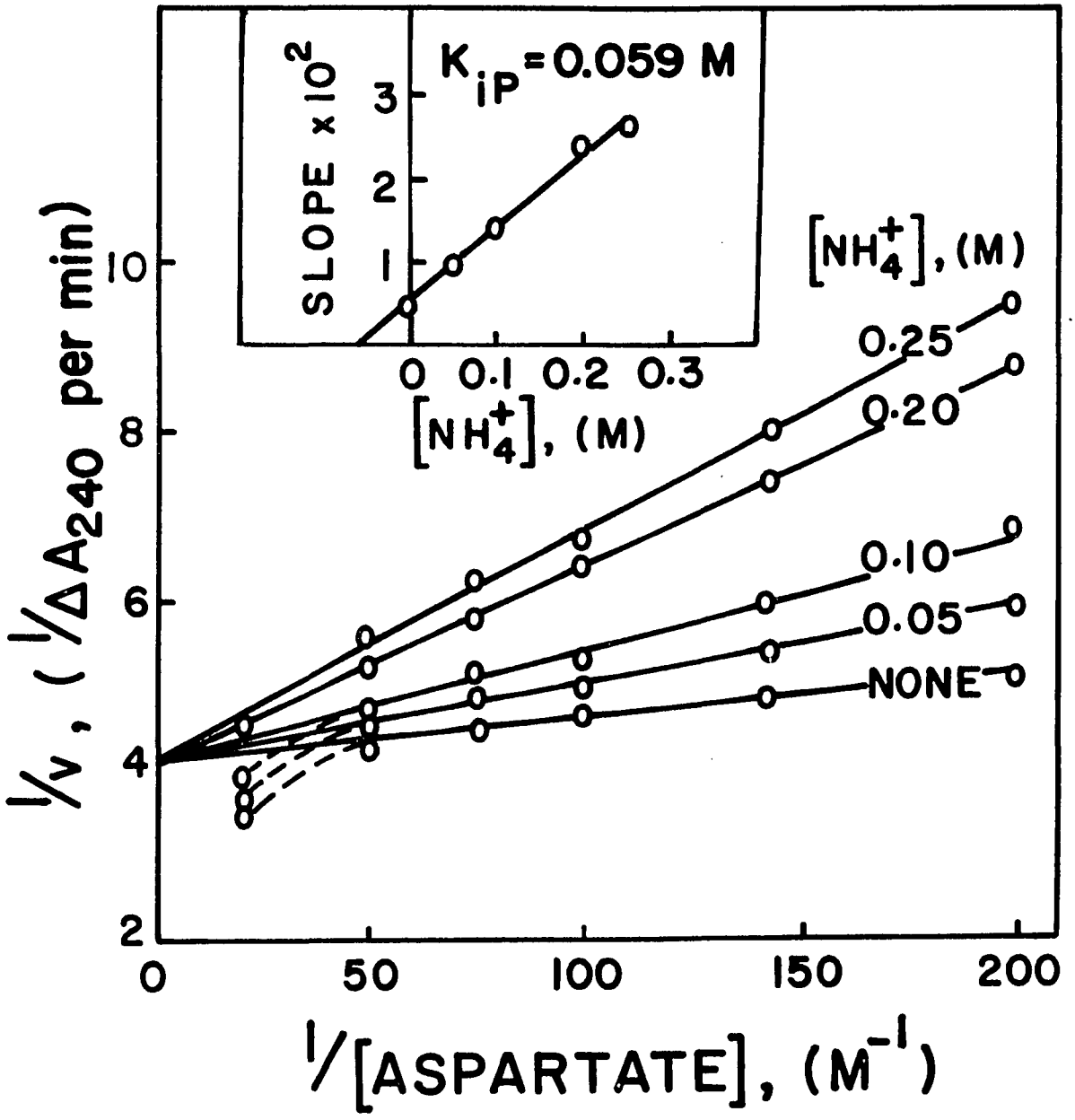
TABLE IV

Comparison of the Valves for $1/v_2$ Determined Experimentally and the Corresponding Values Calculated from the Equation Describing the Amination Reaction (Equation 9). The NH_4^+ and the fumarate concentrations were taken from Figure 5.

$[NH_4^+]$ mM	$1/v_2$ ($1/\Delta A_{240}$ per min) $1/v_2$ Observed		$1/v_2$ Calculated
	[Fumarate]	=	0.15 mM
25.0	37.0		36.5
33.3	30.3		30.2
50.0	23.8		23.4
100.0	17.2		16.9
	[Fumarate]	=	0.25 mM
25.0	30.3		30.7
33.3	25.0		25.3
50.0	19.2		19.7
100.0	13.7		14.2
	[Fumarate]	=	0.50 mM
25.0	27.0		26.3
33.3	22.2		21.7
50.0	17.5		16.9
100.0	11.1		12.2
	[Fumarate]	=	0.70 mM
25.0	25.0		25.0
33.3	20.8		20.7
50.0	15.9		16.1
100.0	9.5		11.6
	[Fumarate]	=	∞
25.0	22.0		21.9
33.3	18.2		18.1
50.0	14.1		14.1

FIGURE 7

Competitive inhibition of the deamination of 2S-aspartate by NH_4^+. Inset: Relationship of the slopes of the primary plots to NH_4^+ concentration. Each reaction mixture contained: 50 mM Tris-HCl, pH 7.0; 50 mM KCl; 1 mM $MgSO_4$; 0.1 mM EDTA; the indicated concentrations of 2S-aspartate and NH_4^+, and 0.3 units of aspartase. Initial velocities were determined at 28° with Gilford Model 2000 recording spectrophotometer.


K_{iP}=0.059 M
SLOPE x 10^2
3
2
1
0 0.1 0.2 0.3
$[NH_4^+]$, (M)
$[NH_4^+]$, (M)
0.25
0.20
0.10
0.05
NONE
1/v, (1/ΔA240 per min)
10
8
6
4
2
0
50
100
150
200
1/[ASPARTATE], (M^{-1})

concentration range of NH_4Cl used. Further evidence for simple linear competitive inhibition was provided by the linear plots of $1/v_1$ *versus* NH_4^+ concentration illustrated in Figure 8. The apparent K_i for NH_4^+ estimated from the intersection of these Dixon plots agreed with the K_i calculated from the slope replot.

As shown in Figure 9, potassium fumarate within the concentration range used, also gave competitive inhibition with 2S-aspartate as substrate. This is in agreement with the earlier data of Sekijo *et al.* (1965). This inhibition was ascertained to be linear from the slope replot given in the inset of Figure 9. Simple competitive inhibition was likewise indicated by the Dixon plots in Figure 10. As expected, the apparent K_i for fumarate determined from the slope replot and from the intersection of the corresponding linear Dixon plots were in agreement.

Kinetic Constants. Values obtained for the apparent Michaelis, dissociation, and inhibition constants of the aspartase reaction, pH 7.0, are listed in Table V. The K_m for 2S-aspartate represents the average calculated from the abscissa intercepts of double-reciprocal plots of aspartate deamination in the absence of product. The K_m's and K_d's for NH_4^+ and fumarate are averages obtained from the initial velocity studies in the direction of fumarate amination. The K_i's are averages obtained from slope replots and Dixon plots derived from the product inhibition patterns. The similarity between the values of K_d and K_i for NH_4^+ or fumarate is not surprising since both constants describe the same interaction between the enzyme and NH_4^+ or fumarate.

FIGURE 8

Dixon plots showing that the inhibition of aspartate deamination by NH_4^+ was of the simple (linear) competitive type within the indicated concentration ranges of 2S-aspartate and NH_4^+. These plots were derived from the data in Figure 7.

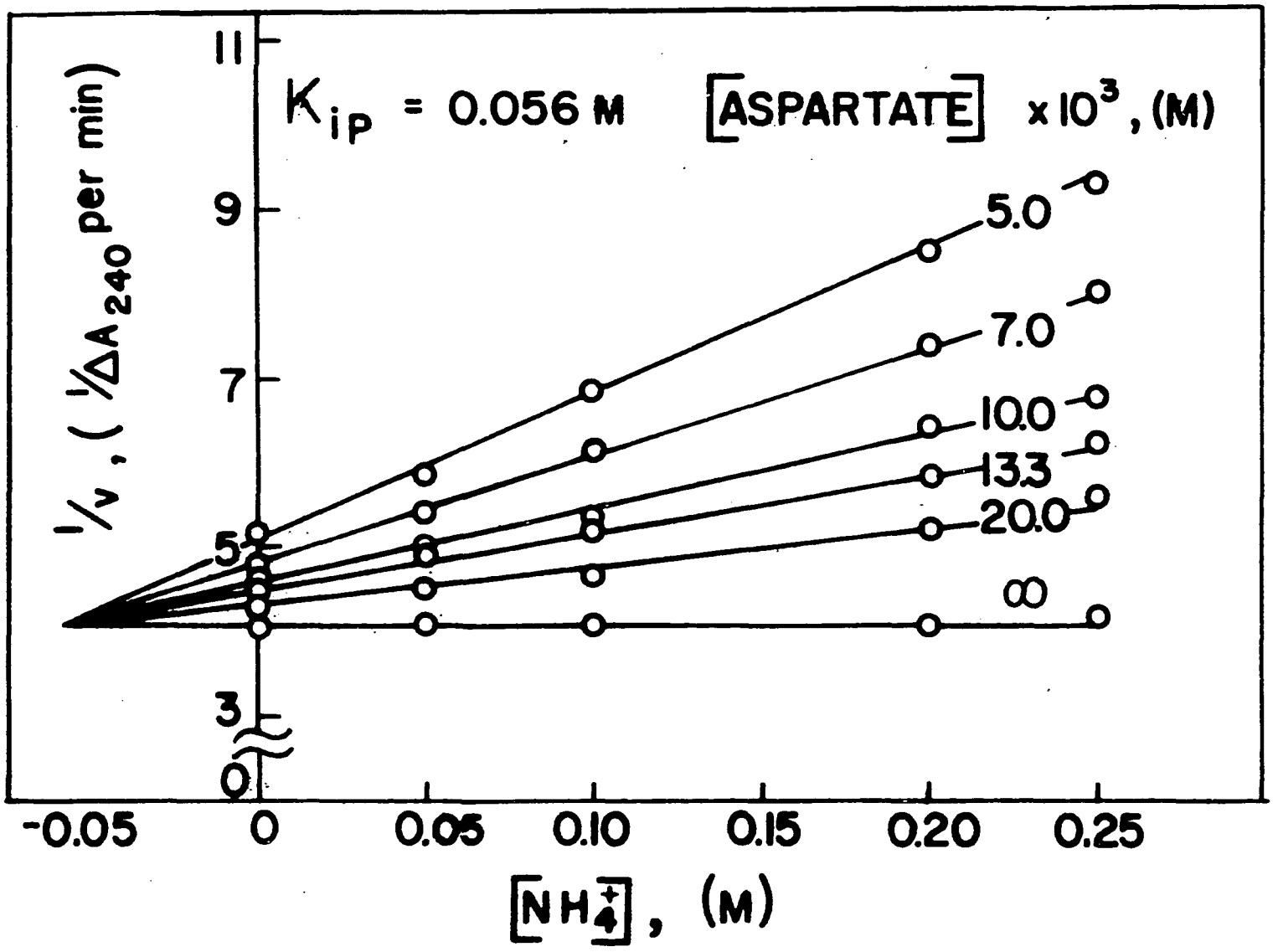

K_{ip} = 0.056 M
[ASPARTATE] x 10^3, (M)
5.0
7.0
10.0
13.3
20.0
∞
1/v, (1/ΔA_{240} per min)
11
9
7
5
3
0
-0.05
0
0.05
0.10
0.15
0.20
0.25
[NH_4^+], (M)

FIGURE 9

Competitive inhibition of the deamination of 2S-aspartate by fumarate. Inset: Relationship of the slopes of the primary plots to fumarate concentration. The composition of each assay solution was identical to that described in Figure 7 except that fumarate was the product. Initial rates were obtained at 26° with a Cary Model 14 recording spectrophotometer. Cuvets containing the standard assay mixture with fumarate at concentrations corresponding to those in the sample cells, but without enzyme, were used as references.

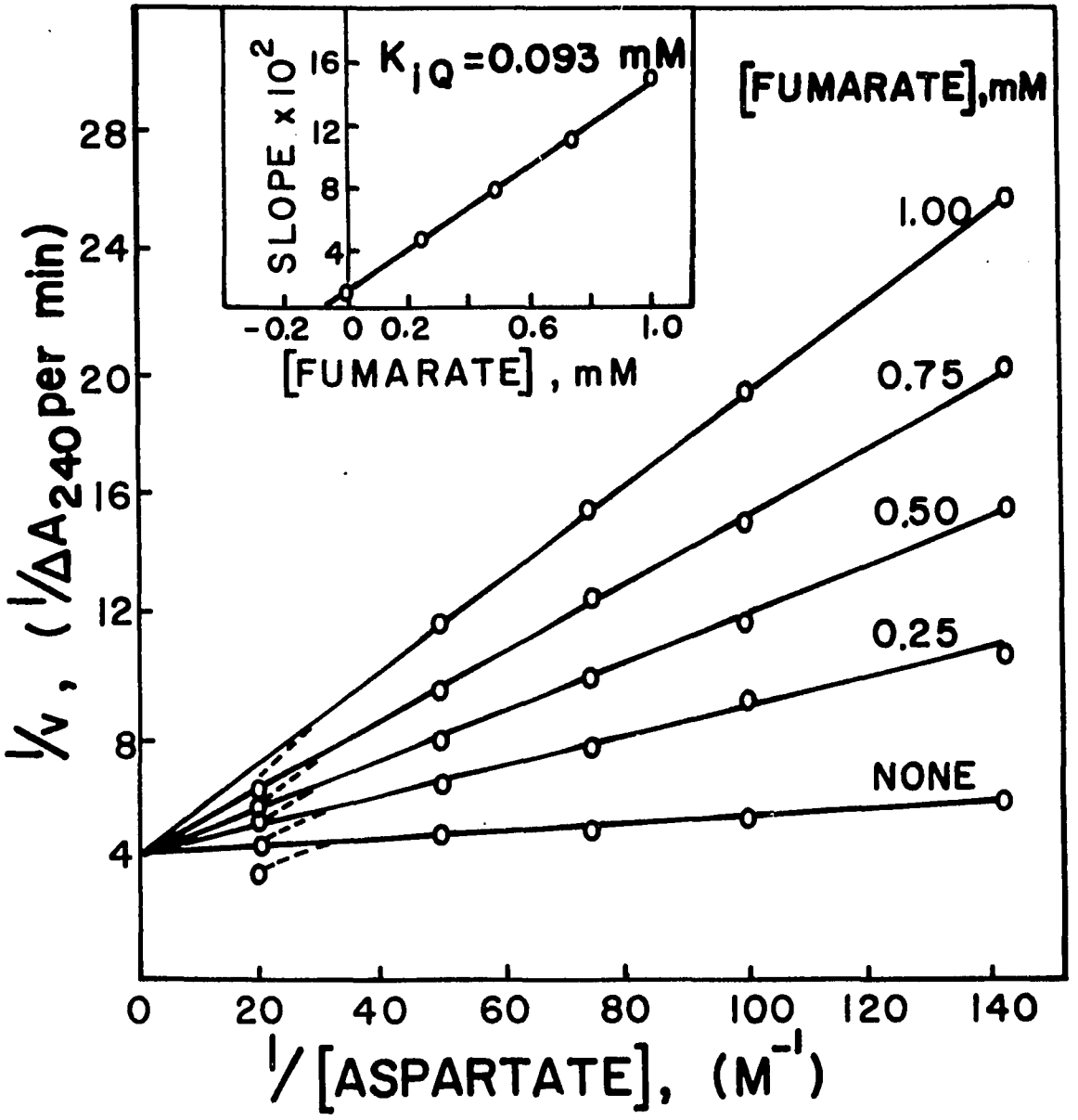

K_{IQ}=0.093 mM
SLOPE x 10^2
16
12
8
4
-0.2
0
0.2
0.6
1.0
[FUMARATE] , mM
[FUMARATE],mM
1.00
0.75
0.50
0.25
NONE
1/v , ($^1/\Delta A_{240}$ per min)
28
24
20
16
12
8
4
0
20
40
60
80
100
120
140
1/ [ASPARTATE], (M^{-1})

FIGURE 10

Dixon plots showing that the inhibition of aspartate deamination by fumarate was of the simple (linear) competitive type within the indicated concentration ranges of 2S-aspartate and fumarate. These plots were derived from the data in Figure 9.

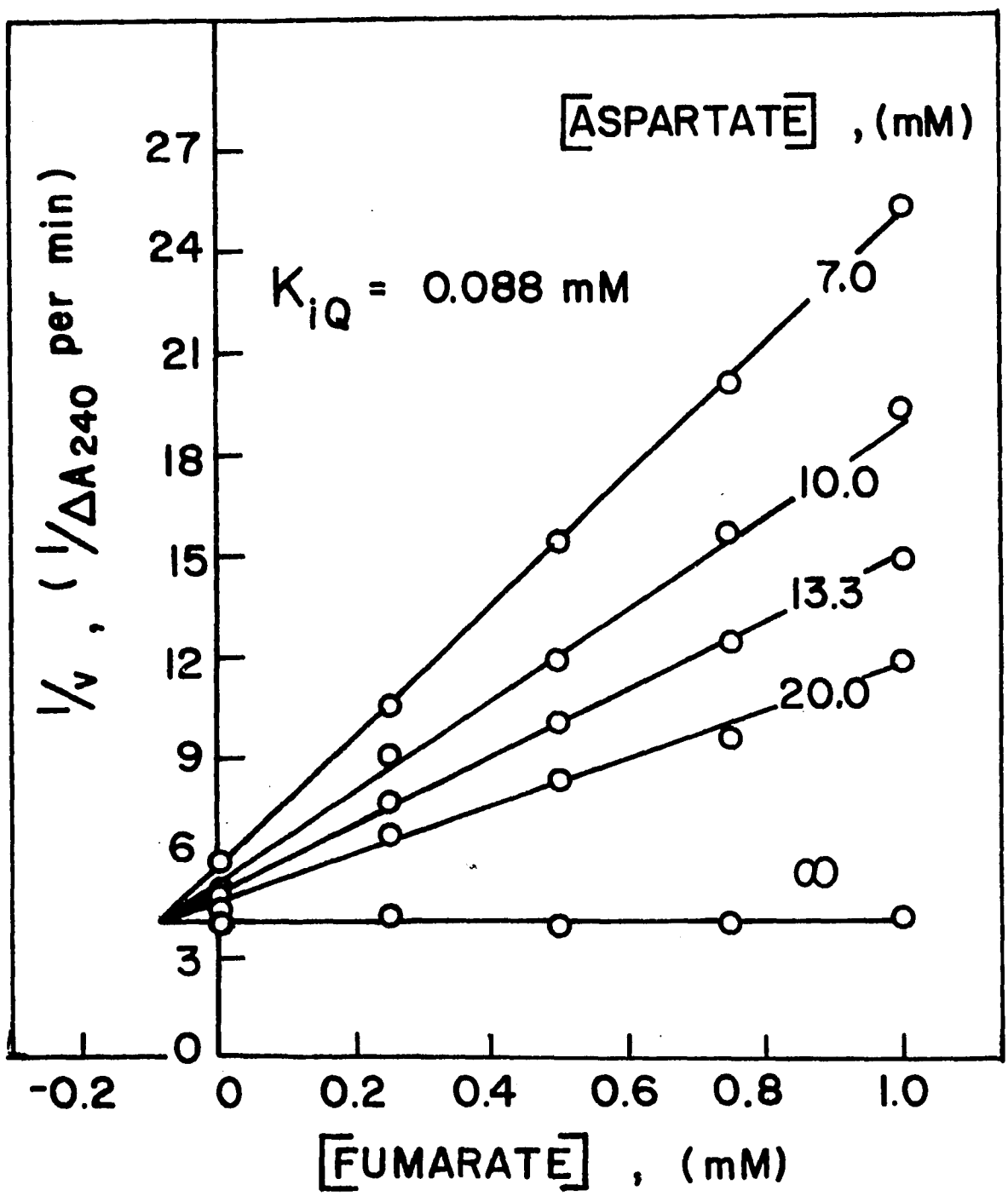

[ASPARTATE] , (mM)
K_{iQ} = 0.088 mM
7.0
10.0
13.3
20.0
∞
1/v , (1/ΔA240 per min)
27
24
21
18
15
12
9
6
3
0
-0.2
0
0.2
0.4
0.6
0.8
1.0
[FUMARATE] , (mM)

TABLE V

Apparent Kinetic Constants for the Aspartase Reaction Determined at pH 7.0 from Initial Velocity Studies of Fumarate Amination and Product Inhibition Studies of 2S-aspartate Deamination. The variations are given in terms of one standard deviation.

Kinetic Constant[a]	From Initial Velocity Studies (mM)	From Product Inhibition Studies (mM)
K_A		2.0 ± 0.9
K_P	63.0 ± 1.7	
K_{dP}	63.0 ± 1.7	
K_{iP}		56.9 ± 2.2
K_Q	0.100 ± 0.003	
K_{dQ}	0.100 ± 0.003	
K_{iQ}		0.089 ± 0.005

[a]Identifying subscripts: A, 2S-aspartate; P, NH_4^+; Q, fumarate.

Isotope Content of Labeled 2S-Aspartic Acid

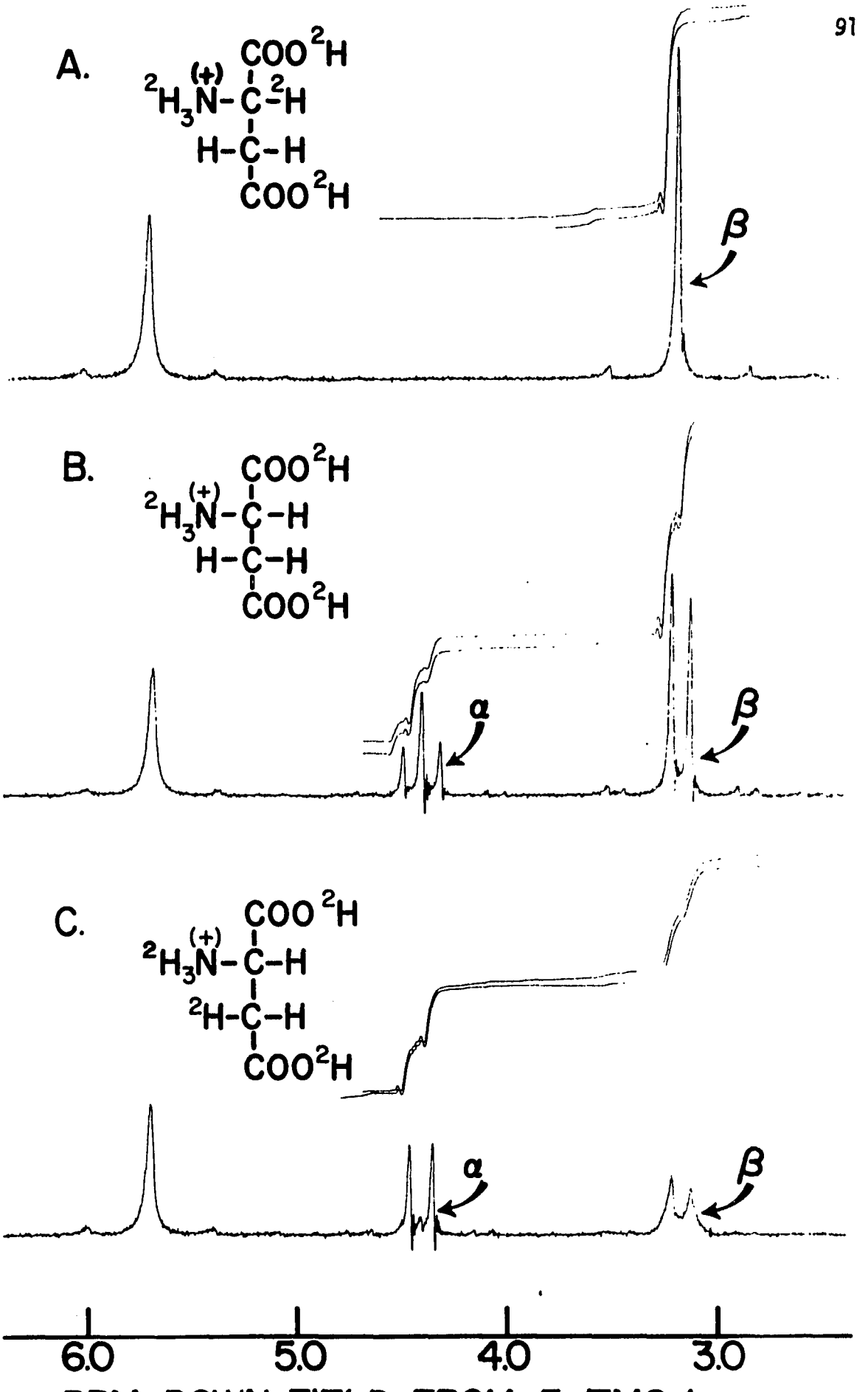
Nuclear Magnetic Resonance Spectroscopy. The NMR spectra of 2S-aspartic acid and the two ^{2}H-labeled forms are shown in Figure 11 (a-c). In the spectrum of 2S-aspartic acid ^{2}HCl (Figure 11b) the triplet located at 4.43 ppm relative to 3-trimethylsilyl-1-propane sulfonate was assigned to the α proton. The doublet at 3.16 ppm was due to the protons at the β carbon; whereas, the water protons gave a signal at 5.74 ppm. The ratio of the area of the β-proton signal to that of the α-proton signal was 2.02. This spectrum of the acid ^{2}HCl in $^{2}H_2O$ containing 3 N [^{2}H] trifluoroacetic acid was less complex than the corresponding one obtained for the potassium salt in $^{2}H_2O$ at pH 7.0. Presumably, deuteration of the carboxylate groups of the acid reduced the electronic repulsions between them thereby allowing greater rotation about the bond between the α and β carbons. As a result, the β-protons became more equivalent in terms of their coupling with the α-proton.

In the spectrum of 2S-[2-^{2}H]aspartic acid ^{2}HCl (Figure 11a), the signal at 3.16 ppm was reduced to a single peak. Moreover, the signal at 4.43 ppm was absent, indicating that replacement of the α-proton by ^{2}H was essentially complete (≥98%). If it is valid to compare the integrated β-proton signal in this spectrum with the same signal in the spectrum of the unlabeled acid, remembering that these spectra were obtained in different NMR tubes, then little or no ^{2}H was incorporated at the β position during synthesis of the labeled acid.

In the spectrum of 2S,3R-[3-^{2}H]aspartic acid ^{2}HCl (Figure 11c), the α- and β-proton signals were doublets. However, the observation of a

FIGURE 11

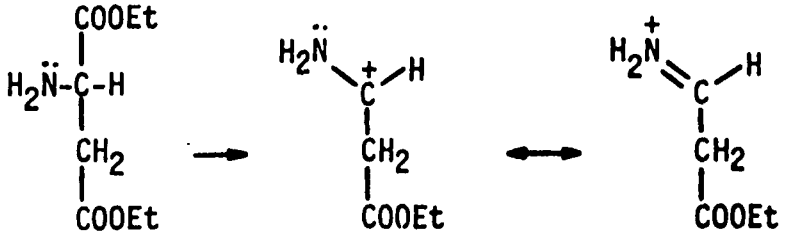
The NMR spectra of (a) 2S-[2-^{2}H]aspartic acid ^{2}HCl, (b) 2S-aspartic acid ^{2}HCl, and (c) 2S,3R-[3-^{2}H]aspartic acid ^{2}HCl. The spectra were determined with a Perkin Elmer R12B (60 MHz) at a sweep range of 5 ppm. The present solvent system of $^{2}H_2O$ made 3 N in [^{2}H]trifluoroacetic acid was similar to that used by Smith and Ihrig (1969) to obtain the spectra of selected amino acids. The method of sample preparation was described in Chapter II.


A.
B.
C.
α
β
6.0
5.0
4.0
3.0
PPM DOWN FIELD FROM 3-TMS-1-PROPANE SULFONATE

small peak in the center of the doublet at 4.43 ppm suggested that ^2H incorporation at the 3R position of the acid was not complete since some splitting, though small, occurred. The equivalency of the areas under the signals at 4.43 ppm for 2S,3R-[3-^2H]aspartic acid and the unlabeled species implied that little, if any, exchange occurred at the α carbon during the enzymatic synthesis of this labeled form.

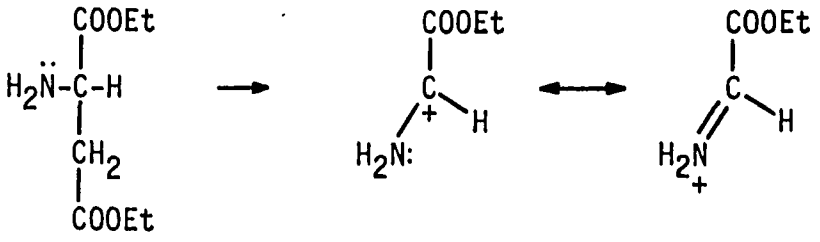
The ^2H enrichments at the α and β positions of the labeled forms of 2S-aspartic acid deduced from the corresponding spectra are given in Table VII.

Mass Spectroscopy. The mass-spectral fragmentation pattern obtained for the diethyl ester hydrochloride of 2S-aspartic acid was identical to that reported by Biemann et al. (1961) for the free ester. Therefore, the same nomenclature and major peak assignments were used here. The peak due to the molecular ion occurred at m/e = 189, and its intensity was quite small which was not uncommon for amino acid derivatives of this type. The most intense peak or "amine" fragment appeared at m/e = 116 and probably resulted from cleavage of the bond between the carboxyl and α carbons. The carbonium ion that formed could have been stabilized by resonance as shown:



The ester fragment of much less intensity was observed at m/e = 102. It

could have arisen as the result of breakage of the bond between the α and β carbons leading also to a resonance-stabilized ion:



Andersson (1958) identified similar fragments in the methyl esters of selected amino acids.

The small peaks observed at m/e = 115 and 114 were presumably due to the loss of one and two hydrogen atoms from the amine fragment. Fortunately, these two signals, when considered together, were less than 3% of the relative intensity of the amine fragment. Finally, the small signals at m/e = 117 and 118 resulted from the presence of the natural abundances of ^{2}H, ^{13}C, ^{15}N, and ^{18}O in this fragment.

Table VI includes the relative intensities of the molecular ion, the amine fragment, the ester fragment, and associated peaks found in the spectra of the labeled and unlabeled diester derivatives. The cracking patterns of all the derivatives were essentially identical except that in the patterns of the labeled compounds, the positions of the amine fragment, the molecular ion, and, in some cases, the ester fragment increased one m/e. The ester fragment for the β-^{2}H derivative remained at m/e = 102 implying the absence of ^{2}H at the α carbon.

Although either the ester or the amine fragment could be used to estimate the isotope enrichment in the α-labeled compounds, the latter was preferred since it was more intense. Furthermore, it was difficult

TABLE VI

Relative Intensities of the Molecular Ion, the "Amine" Fragment, the "Ester" Fragment, and Associated Peaks Observed in the Mass Spectra of the Unlabeled and the Three Labeled Diethyl Ester Hydrochlorides of 2S-aspartic Acid. The amine fragment, the most intense peak, was assigned a value of 100. In the Hatachi-Perkin Elmer RMS-4 mass spectrometer, the sample and chamber temperatures were generally maintained at 90°-100° and 70°-80°, respectively.

Relative Intensities of Peaks for the Labeled Aspartic Acid Diethyl Ester Hydrochlorides

Fragment (m/e)	2S-Aspartic Acid	2S-[2-^{15}N]-Aspartic Acid	2S-[2-^{2}H]-Aspartic Acid	2S,3R-[3-^{2}H] Aspartic Acid
Ester				
100	0.3	0.4	0.5	0.3
101	0.5	0.6	0	0.3
102	15.9	0.9	0.9	19.3
103	1.0	17.4	16.3	1.5
104	0.2	1.0	1.3	1.5
105	0.5	1.0	0.5	0
Amine				
114	0.8	0	0	0
115	2.1	0.9	1.2	0.9
116	100.0	6.3	3.1	13.0
117	6.3	100.0	100.0	100.0
118	0.8	6.3	7.2	6.3
119	0	0.8	1.3	0.7
Molecular				
189	0.6	0	0	0
190	0.3	0.6	1.0	2.5
191	0	0.3	0.8	0.3

to tell if the small signals next to the ester fragment were due to that fragment's loss of hydrogen. In determining the isotope enrichments, it was necessary to assume that isotope effects on the cracking patterns were inconsequential. Since the peak at m/e = 116 for each labeled diester was produced not only by the unlabeled fragment present but by the labeled fragment minus one hydrogen as well, reliable estimates were obtained only if the contribution from the latter fragment was removed. This was effectively accomplished with the following set of simultaneous equations:

$$\begin{aligned} 0.0212X + Y &= S \\ X + 0.0634Y &= 100 \end{aligned} \qquad (10)$$

In these equations, X represented the contribution of the labeled fragment to the relative intensity of the peak at m/e = 117 (S + 1 peak set to 100) and Y was the contribution of the unlabeled fragment to that at m/e = 116 (S peak). The term, 0.0212X, was the contribution to the S peak made by the enriched fragment minus one hydrogen; whereas, 0.0634Y was the contribution to the S + 1 peak made by the unlabeled fragment containing the natural abundance of ^{2}H, ^{13}C, or ^{15}N. The coefficients for these last two terms were easily obtained from signals associated with the amine fragment in the spectrum of the unlabeled diester. The value found for the contribution of the unlabeled fragment to the peak at m/e = 116 in the spectrum of each labeled diester was then used in Equation 3 (Chapter II) to calculate the isotope enrichment.

The enrichments estimated for the labeled derivatives are included in Table VII. Also given is the ^{15}N content of 2S-[2-^{15}N]aspartic acid

TABLE VII

Occurrence of ^{2}H or ^{15}N in the Labeled Forms of 2S-Aspartic Acid as Determined by NMR and Mass Spectroscopy. Details for estimating the various quantities are outlined in this chapter and in Chapter II.

Labeled Aspartic Acid Tested	Location of Isotope	Method and Derivative Used (Values in Atom % Isotope)[a] NMR; Acid ^2HCl	Mass Spec.; Diethyl Ester HCl	Mass Spec.; N_2
2S-[2-^{2}H] Aspartic Acid	α	≥ 98	99.4 ± 0.6	
	β	≤ 2	≤ 1	
2S,3R-[3-^{2}H] Aspartic Acid	α	≤ 2	≤ 2	
	β	92 ± 2	89.4 ± 0.7	
2S-[2-^{15}N] Aspartic Acid	α		96.8 ± 0.7	96.6 ± 0.2

[a]The variations, when determined, are given in terms of one standard deviation.

determined as $[^{15}N]N_2$ by residual gas analysis. The excellent agreement between the two values for the ^{15}N enrichment attests to the validity of assuming that the value estimated from the amine fragment represented the enrichment in the original labeled aspartic acid. The ^{2}H content of the ester fragment of the β-^{2}H derivative was found to be $\leq$ 2 atom % ^{2}H which was good evidence that the aspartase preparations used to synthesize this labeled amino acid were free of contaminating transaminases. If any incorporation of ^{2}H had occurred at the β-position during synthesis of 2S-[2-^{2}H]aspartic acid, then there would have been a high probability of encountering some doubly-labeled fragment at m/e = 118 in the spectrum of that derivative. Accordingly, if the signal at m/e = 117 in the same spectrum was assumed to be equivalent to that at m/e = 116 in the spectrum of the unlabeled diester, then Equation 3 was used to estimate the abundance of doubly labeled fragment. Here, ΔI was the difference between the S + 1 and S + 2 peaks in the two spectra. Less than 1 atom % ^{2}H of additional label was found which implied that little or no β exchange in the amino acid had been catalyzed by GOT. The above results agreed strongly with the enrichment data obtained from the NMR spectra of the labeled amino acids.

Kinetic Isotope Effects

The direct comparison of the initial rates of the reaction using the labeled and unlabeled forms of 2S-aspartate as substrates was justified since the corroborative data from the NMR and mass-spectral studies

implied that the essentially complete incorporation of isotope in each labeled acid was confined to a stereospecific location. Unfortunately, when 2S-[2-^{15}N]aspartate and 2S-aspartate were compared, the results were inconclusive. In some cases the initial deamination rates for the ^{15}N-labeled acid were significantly less, but in others, the differences were not significant. This was most likely due to the error associated with the spectrophotometric method as was suggested by the observation of an average value of 1.03 $\pm$ 0.04 for the ratio, v_{14_N}/v_{15_N}.

Even if the ^{15}N were involved in the rate-limiting step of the reaction, this approach probably was not sensitive enough to detect the expected small isotope effect with any degree of confidence.

The use of ^{2}H in such studies was more desirable because the relative mass difference between ^{2}H and ^{1}H was much greater. Therefore, larger rate differences were expected if the isotope were involved in the rate-determining step. However, with 2S,3R-[3-^{2}H]aspartate as substrate, the maximal velocity for its deamination was not significantly less than that for 2S-aspartate. On the other hand, as shown by the double-reciprocal plots in Figure 12, significantly slower rates were obtained with the 2S-[2-^{2}H]aspartate than with the unlabeled acid. This rate difference was interpreted as an apparent secondary isotope effect of 1.11 $\pm$ 0.02.

A secondary isotope effect was also observed during the deamination of 2S-[2-^{3}H]aspartate. As seen in Figure 13, the discrimination against the ^{3}H-labeled species was reflected in the increase in the specific radioactivity of the fumarate formed in the first 10 min of the reaction. This interpretation was reasonable since as the concentration of the

FIGURE 12

Double-reciprocal plots of the initial rate of fumarate production as a function of 2S-aspartate and 2S-[2-^{2}H]aspartate concentrations. Each reaction solution contained in a total volume of 3 ml: 50 mM Tris HCl buffer, pH 7.0; 1 mM $MgSO_4$; 0.1 mM EDTA, and the indicated range of substrate concentrations. Initial rates were determined at 28° in the Gilford Model 2000 recording spectrophotometer.

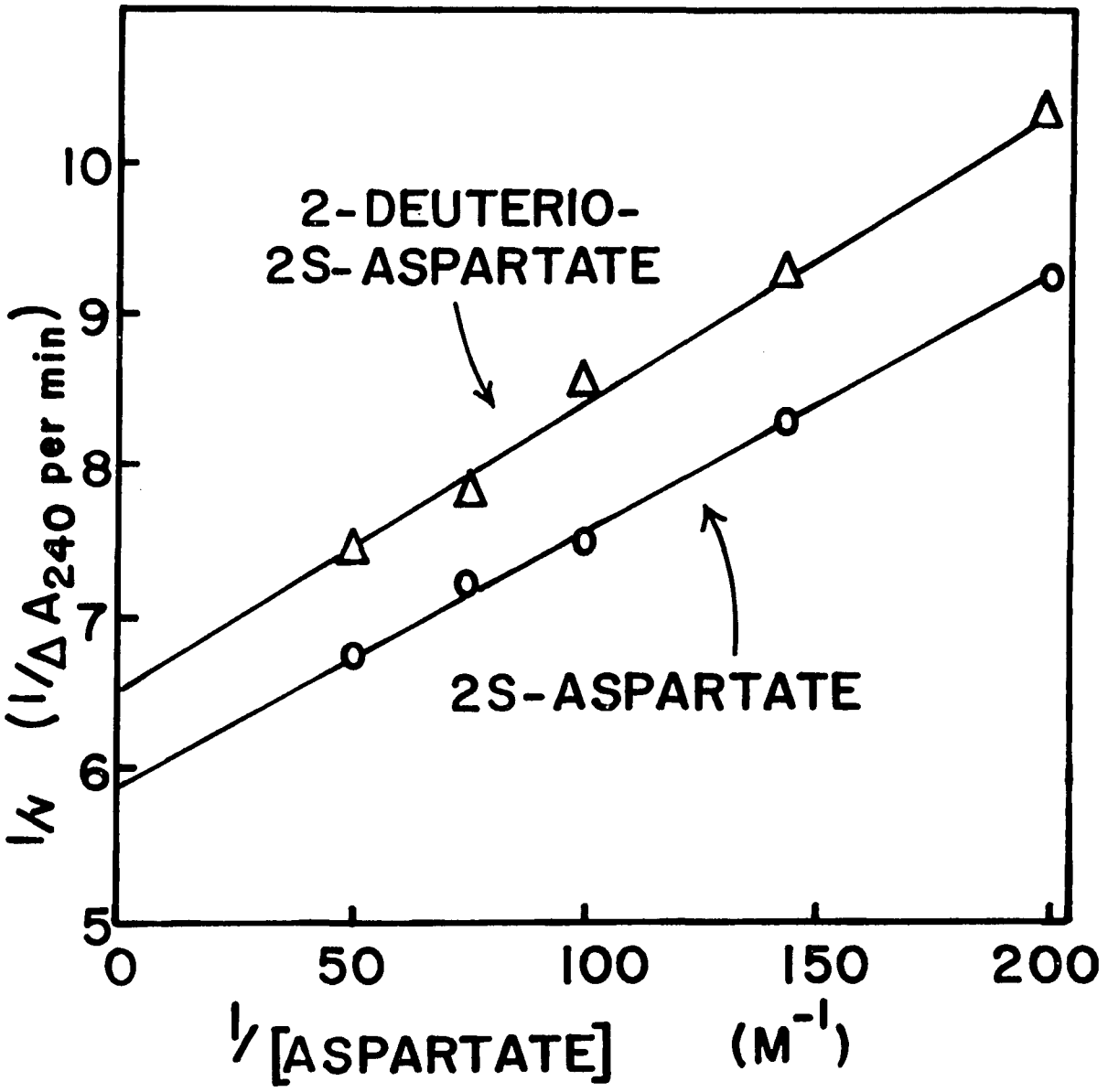
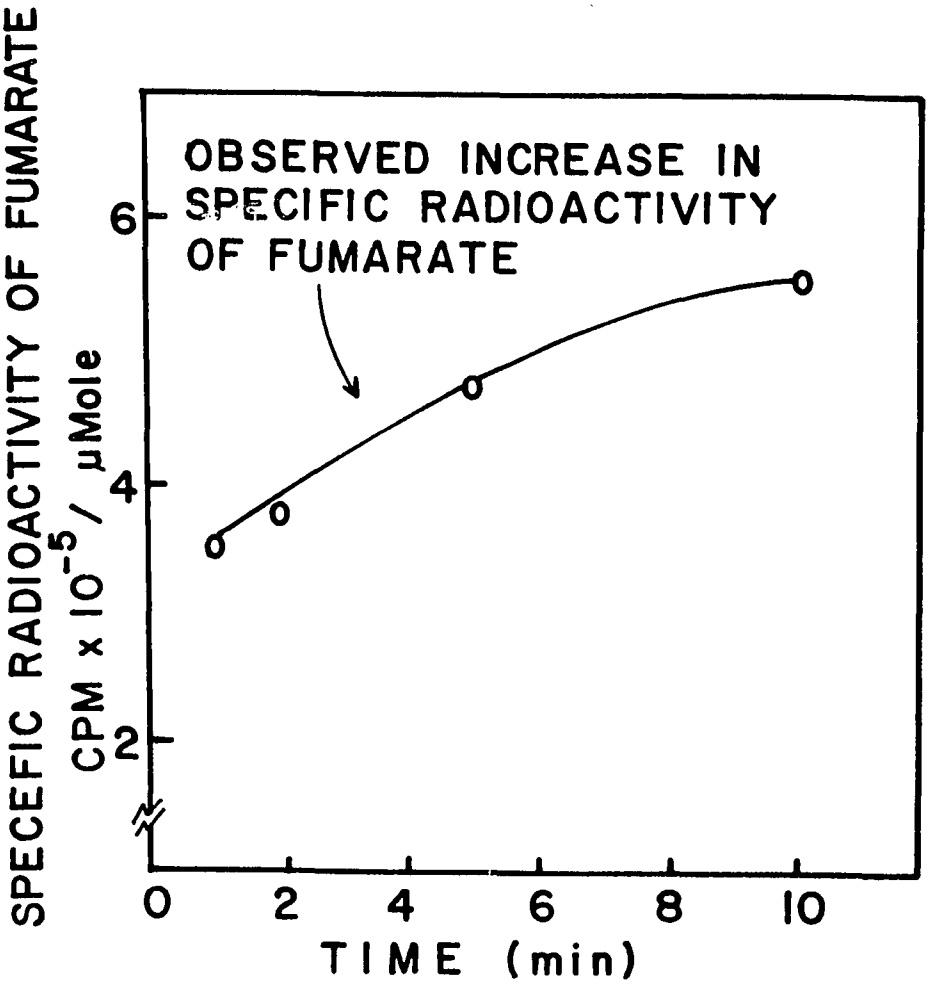

2-DEUTERIO-
2S-ASPARTATE
2S-ASPARTATE
1/v (1/Δ A240 per min)
1/[ASPARTATE] (M⁻¹)
5
6
7
8
9
10
0
50
100
150
200

FIGURE 13

Secondary isotope effect in the conversion of 2S-[2-^{3}H]aspartate to [^{3}H]fumarate. The incubation solution was formulated to contain in a final volume of 20 ml: 50 mM potassium phosphate buffer, pH 7.0; 13.1 mM potassium 2S-[2-^{3}H]aspartate; 1.5 mM $MgSO_4$; 150 μM EDTA; and 2 ml of aspartase (4 units). The preparation of samples for ^{3}H and fumarate analyses is described in Chapter II.


OBSERVED INCREASE IN
SPECIFIC RADIOACTIVITY
OF FUMARATE
SPECEFIC RADIOACTIVITY OF FUMARATE
CPM x 10⁻⁵ / µMole
6
4
2
0
2
4
6
8
10
TIME (min)

labeled aspartate was increased relative to the unlabeled form, the former reacted faster which resulted in the production of more labeled fumarate relative to its unlabeled counterpart. Otherwise, a constant specific radioactivity for the fumarate would have implied no secondary isotope effect.

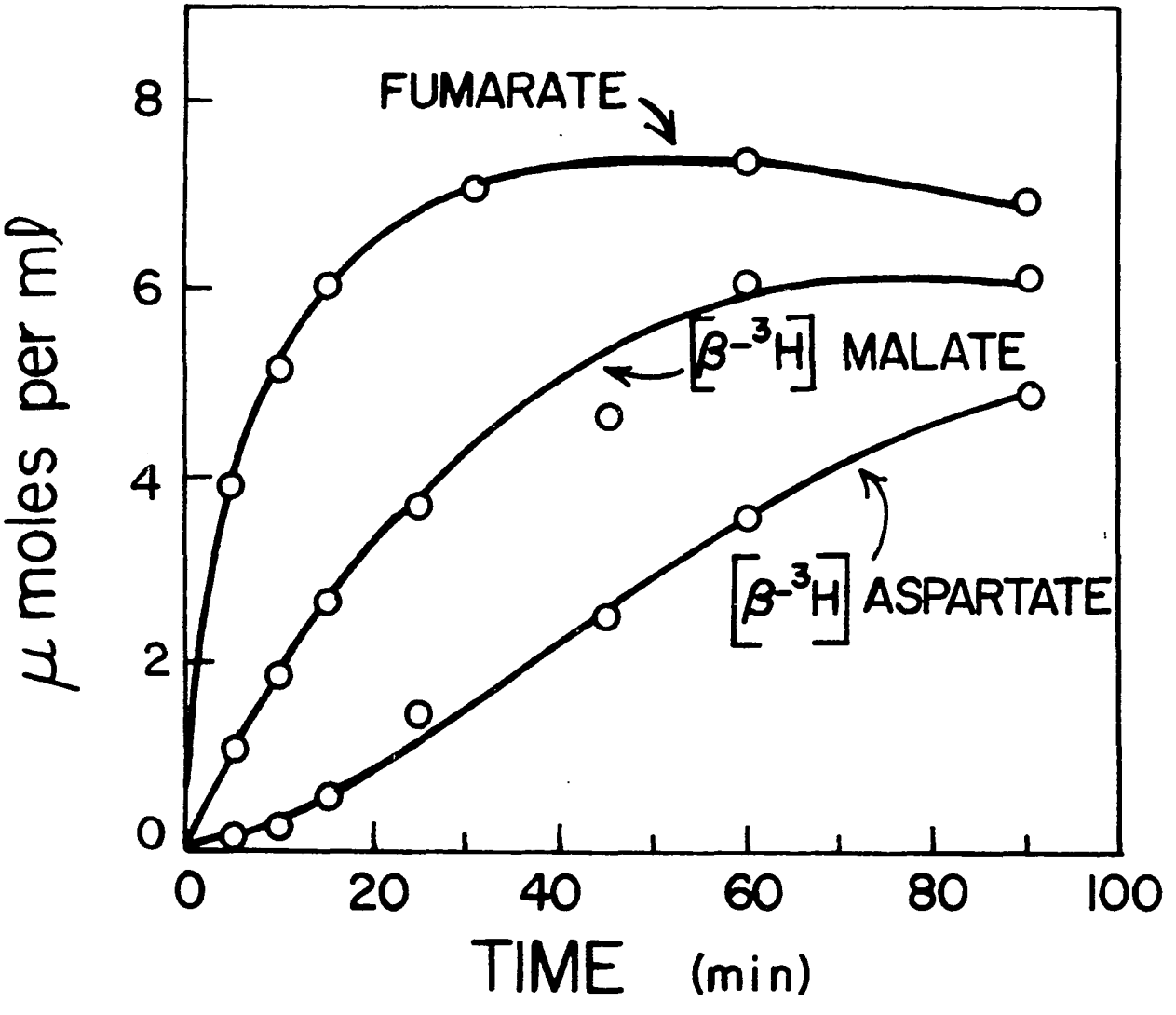
Exchange Studies with ^{3}H, ^{14}C, and ^{15}N

2S-Aspartate Deamination in the Presence of Tritiated Water. As discussed in Chapter II, a preliminary NMR study showed that the incorporation of ^{2}H into unreacted 2S-aspartate was negligible during the initial stages of the forward reaction catalyzed by aspartase. As illustrated in Figure 14, the same conclusion was reached with the more sensitive exchange technique involving the deamination of 2S-aspartate in the presence of tritiated water. The detectable incorporation of ^{3}H into aspartate in the early stages of the reaction was much less than the production of fumarate. The formation of ^{3}H-labeled malate implied the presence of fumarase in the reaction mixture. Although the concentration of fumarase was considerably less than that of aspartase, the appearance of label into malate was still faster than its exchange into unreacted aspartate. This slow exchange of ^{3}H into 2S-aspartate was in agreement with that expected if the sole route of ^{3}H incorporation was through the amination of fumarate. A similar finding was obtained by Englard (1958) from mechanistic studies of the amination reaction in $^{2}H_{2}O$.

Exchange of ^{14}C and ^{15}N at Chemical Equilibrium. The exchange rates of ^{14}C and ^{15}N from [1,4-^{14}C]fumarate and [^{15}N]NH_4^+ respectively, into

FIGURE 14

Incorporation of ^{3}H into 2S-aspartate and 2S-malate from tritiated water during the deamination reaction catalyzed by an aspartase preparation contaminated with fumarase. The formulation of the incubation mixtures and the preparation of samples for ^{3}H and fumarate determinations are described in Chapter II.


FUMARATE
[β-3H] MALATE
[β-3H] ASPARTATE
μ moles per ml
TIME (min)
0
2
4
6
8
0
20
40
60
80
100

2S-aspartate were determined under conditions in which all the reactants were present at equilibrium concentrations. Since any carbon or nitrogen isotope effect was expected to be very small, the exchange rates obtained with the heavier isotopes should closely approximate the corresponding rates due to the lighter isotopes in the unlabeled reactants.

When $[^{15}N]NH_4^+$ and [1,4-^{14}C]fumarate were at equimolar concentrations, the isotope exchange curves shown in Figure 15 were observed. The data are also presented according to the preferred conventions of Hansen (1968) in Figure 16. The isotope exchanges are expressed in terms of their fractional approach to isotopic equilibrium as a function of time. Under the conditions in which the concentrations of $[^{15}N]NH_4^+$ and [1,4-^{14}C]fumarate were in the region of their K_m values, the curves given in Figure 17 were obtained. The results are also reproduced in terms of the fractional approach to isotopic equilibrium as a function of time in Figure 18. Table VIII below lists the exchange rates of the two isotopes found by multiplying the appropriate concentration factors from Equation 8 by the rate constants predicted by Equations 6 and 7 for each set of reactant concentrations. When the exchange rates for each isotope were calculated at the individual times using the experimental values of F_i (Equation 8), the resulting set agreed within 3 to 5%. Furthermore, the average of these exchange rates was essentially the same as the corresponding rate determined with the aid of Equation 6 or 7.

FIGURE 15

The aspartase-catalyzed exchanges of ^{14}C and ^{15}N in the presence of 122.3 mM 2S-aspartate; 25.0 mM $[^{15}N]NH_4^+$, and 25.0 mM [1,4-^{14}C]fumarate. Besides the reactants, each incubation solution contained in a total volume of 10 ml at 28°: 50 mM Tris HCl, pH 7.0; 1 mM $MgSO_4$; 0.1 mM EDTA; 1 mM mercaptoethanol, and 300 μg aspartase. The preparation of samples for the assay of ^{14}C and ^{15}N is described in Chapter II.

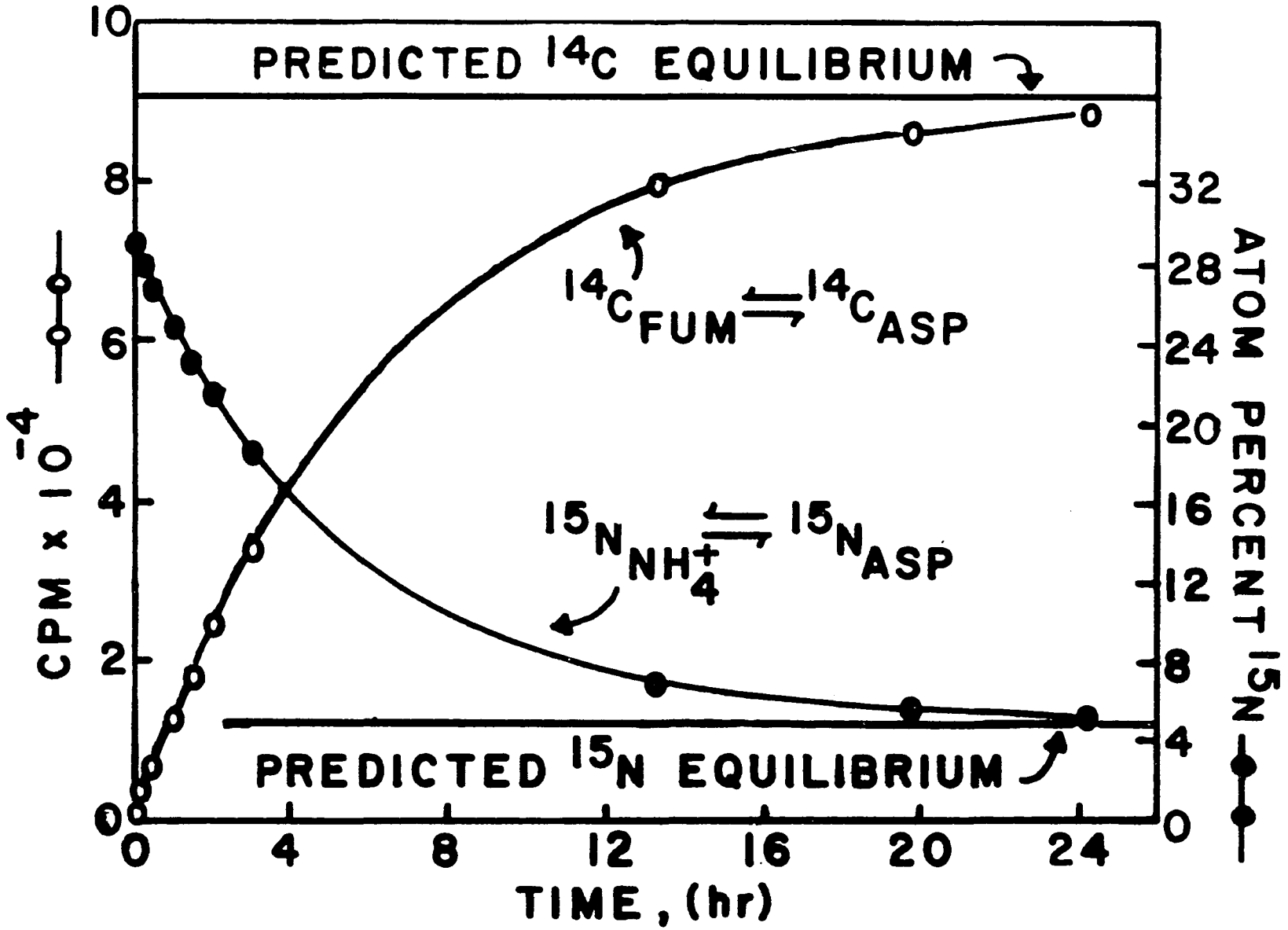

PREDICTED ^{14}C EQUILIBRIUM
$^{14}C_{FUM} \rightleftharpoons {}^{14}C_{ASP}$
$^{15}N_{NH_4^+} \rightleftharpoons {}^{15}N_{ASP}$
PREDICTED ^{15}N EQUILIBRIUM
CPM x 10^{-4}
ATOM PERCENT ^{15}N
TIME, (hr)
0
2
4
6
8
10
0
4
8
12
16
20
24
32
28
24
20
16
12
8
4
0

FIGURE 16

The ^{14}C and ^{15}N exchanges from Figure 15 reproduced in terms of the fractional approach to isotopic equilibrium as a function of time. The values of F_i used to construct these curves were obtained from the relationship:

$$F_i = 1 - e^{-kt_i}$$

in which the k's were predicted with Equations 6 and 7. The experimental values of F_i were based on the predicted equilibrium distribution of the corresponding isotopes.

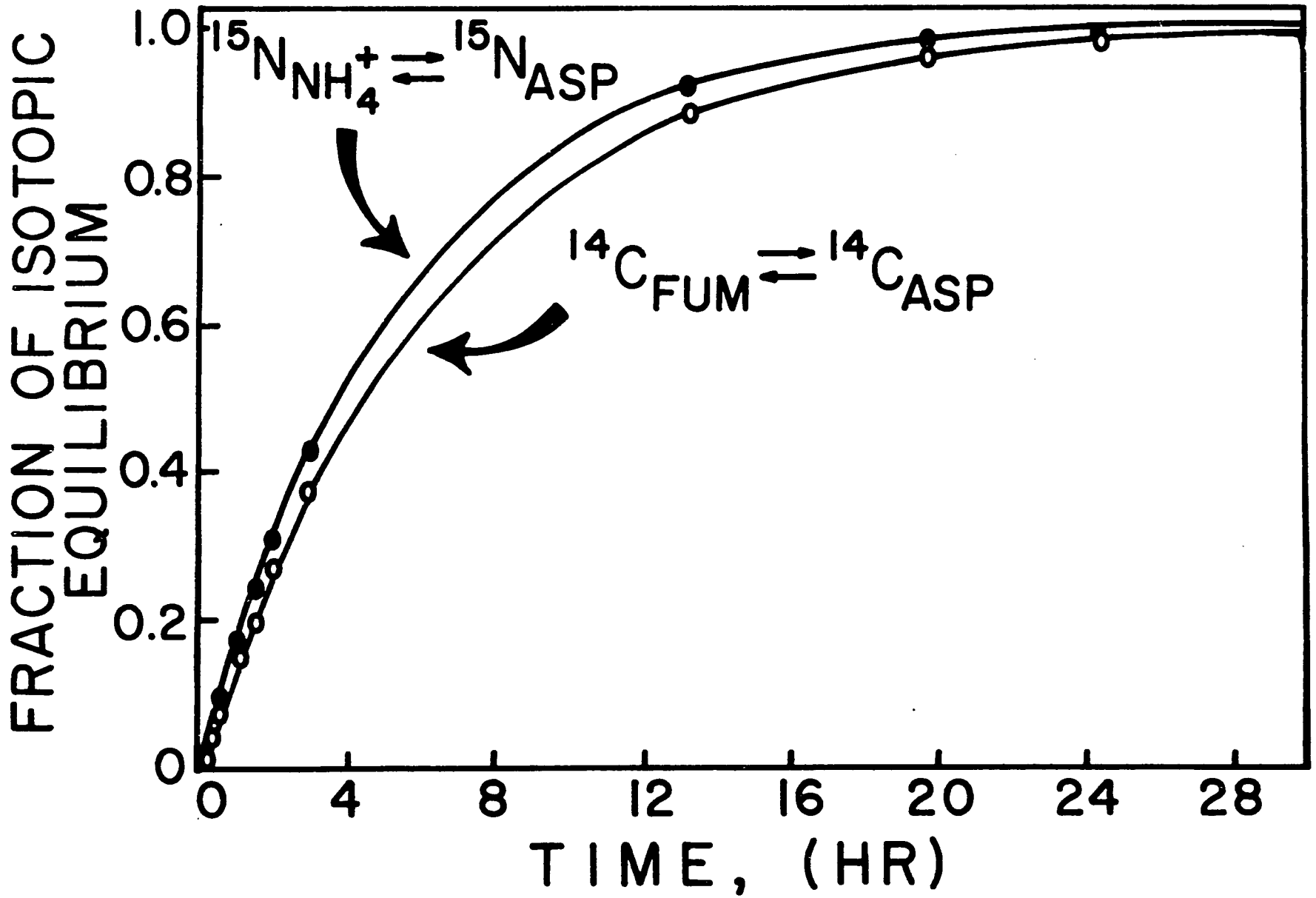

FRACTION OF ISOTOPIC EQUILIBRIUM
1.0
0.8
0.6
0.4
0.2
0
$^{15}N_{NH_4^+} \rightleftharpoons {}^{15}N_{ASP}$
$^{14}C_{FUM} \rightleftharpoons {}^{14}C_{ASP}$
0
4
8
12
16
20
24
28
TIME, (HR)

FIGURE 17

The aspartase-catalyzed exchanges of ^{14}C and ^{15}N in the presence of 36.0 mM 2S-aspartate; 61.3 mM $[^{15}N]NH_4^+$, and 3.0 mM [1,4-^{14}C]fumarate. With the exception of the reactant concentrations, the incubation solutions at 28° were identical to those in Figure 15. The preparation of samples for the assay of ^{14}C and ^{15}N is described in Chapter II.

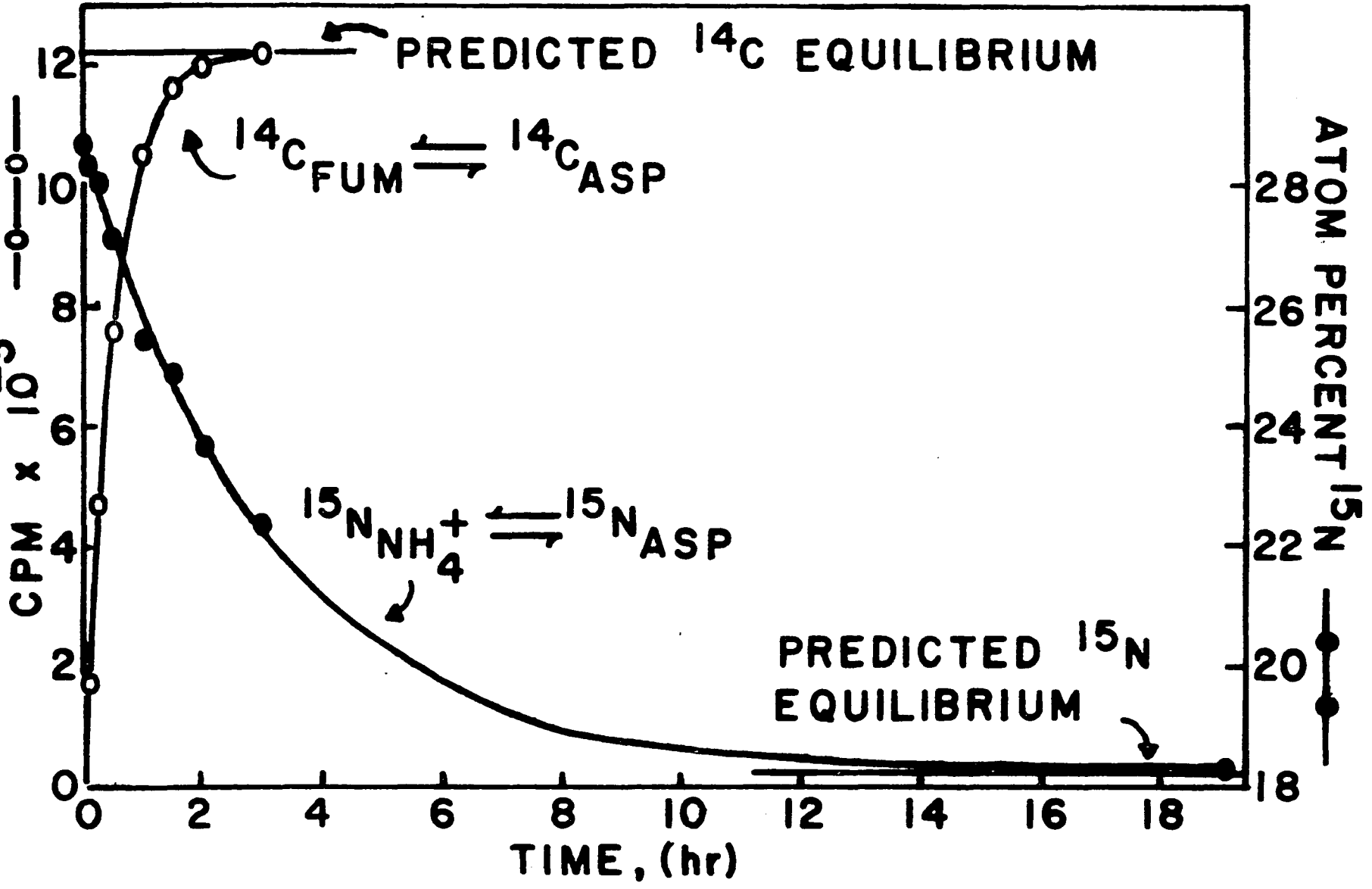

PREDICTED ^{14}C EQUILIBRIUM
$^{14}C_{FUM} \rightleftharpoons {}^{14}C_{ASP}$
$^{15}N_{NH_4^+} \rightleftharpoons {}^{15}N_{ASP}$
PREDICTED ^{15}N EQUILIBRIUM
CPM x 10^{-3}
ATOM PERCENT ^{15}N
TIME, (hr)
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28

FIGURE 18

The ^{14}C and ^{15}N exchanges from Figure 17 expressed in terms of the fractional approach to isotopic equilibrium as a function of time. The predicted and the experimental values of F_i were determined as in Figure 16.

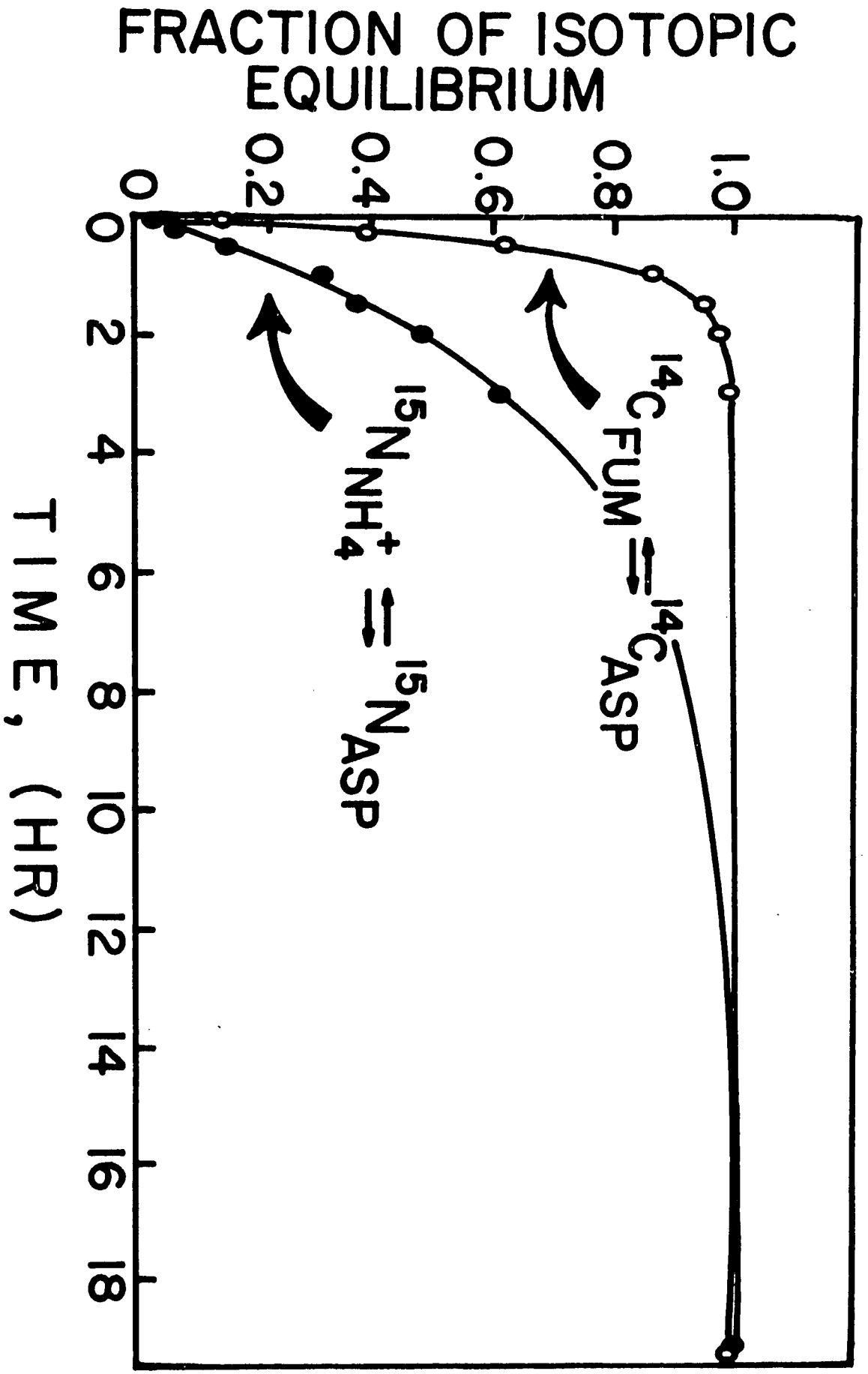

FRACTION OF ISOTOPIC EQUILIBRIUM
0
0.2
0.4
0.6
0.8
1.0
0
2
4
6
8
10
12
14
16
18
TIME, (HR)
$^{14}C_{FUM} \rightleftharpoons {}^{14}C_{ASP}$
$^{15}N_{NH_4^+} \rightleftharpoons {}^{15}N_{ASP}$

TABLE VIII. The Rates of Exchange of ^{14}C and ^{15}N from $^{15}NH_4^+$ and [1,4-^{14}C]Fumarate Respectively, into 2S-Asparate at the Indicated Equilibrium Concentrations of the Reactants.

Equilibrium Concentration of Reactants (mM)[a]			Isotope Exchange Rate (mM/hr)		Ratio $^{15}N/^{14}C$
A	P	Q	^{15}N	^{14}C	
122.3	25.0	25.0	3.87	3.23	1.20
36.0	61.3	3.0	7.35	5.34	1.38

[a]A, P, and Q refer to 2S-aspartate, [^{15}N]NH_4^+, and [1,4-^{14}C]fumarate, respectively.

Under both sets of conditions, the nitrogen exchange was more rapid than the carbon exchange. When the concentrations of [^{15}N]NH_4^+ and [1,4-^{14}C]fumarate were both 25.0 mM, the rate of the ^{15}N exchange was 20% greater than the corresponding ^{14}C exchange. However, when the [^{15}N]NH_4^+ concentration was increased with a simultaneous decrease in the [1,4-^{14}C]fumarate concentration, the rates of both exchanges were increased, although in this instance, the nitrogen exchange was 38% more rapid. Recall from the initial velocity studies (Figure 5) that the reciprocal of the initial velocities found for the different levels of fumarate deviated from linearity at the highest NH_4^+ concentration used. Possibly, the increase in the [^{15}N]NH_4^+ concentration in the present situation may have produced a similar cooperative effect on the exchange reactions.

IV. DISCUSSION

Purification of Aspartase

Although the yield was no greater than that reported by Williams and Lartigue (1967, 1969), the specific activity of the aspartase prepared by the present procedure was nearly doubled. Since the primary objective of the experiments described in Chapters II and III was to investigate the enzyme's mechanism of action, no critical studies were carried out toward the development of a superior purification scheme or to stabilize the resulting preparations. Nevertheless, it is of interest to compare this procedure with others that have recently appeared in the literature. The scheme of Rudolph and Fromm (1971) involved a similar strategy in that the crude enzyme obtained by treating lysed *E. coli* cells with strepomycin sulfate, low pH, and $(NH_4)_2SO_4$ was further purified by chromatography on DEAE-cellulose and Sephadex G-100. In the final step, the pooled aspartase-containing fractions were concentrated and extracted with $(NH_4)_2SO_4$. Their procedure, however, was not any shorter and, in fact, included three dialysis steps. Likewise, that of Suzuki *et al.* (1973) offered no additional advantages. In brief, their scheme consisted of sonication; treatments with streptomycin sulfate, heat, $(NH_4)_2SO_4$, and calcium phosphate gel; and chromatography on columns of DEAE-Sephadex A-50, hydroxylapatite, and Sepharose 6B. Furthermore, at least three overnight dialyses and seven different precipitations with $(NH_4)_2SO_4$ were involved. In spite of the apparent homogeneity of the enzyme isolated by both procedures, the specific activity was not substantially

increased over that of the E. aerogenes aspartase.

The removal of fumarase from aspartase was of utmost importance, especially if reliable conclusions were to be drawn from the results of exchange studies involving lengthy incubation times. The fumarase from pig heart possesses a K_m for fumarate that is approximately two orders of magnitude less than the corresponding K_m of aspartase from E. aerogenes. If the bacterial fumarase were similar kinetically to the pig heart enzyme, then contamination of aspartase preparations by only a small amount of the bacterial fumarase would have led to significant interferences. Fortunately this problem has been eliminated by the successful separation of fumarase from aspartase by hydroxylapatite chromatography. However, in neither of the reports by Rudolph and Fromm (1971) or Suzuki et al. (1973) did the authors clearly state that their preparations from E. coli were free of fumarase.

Just recently, Tosa et al. (1974) clearly demonstrated the separation of fumarase from E. coli aspartase by an approach based on affinity chromatography. When a fumarase-contaminated preparation was applied to a column of N-(ω-aminohexyl)-2S-aspartic acid Sehparose 6B, aspartase bound to the attached amino acid, and fumarase washed through. Moreover, the possibility exists that through the use of appropriate gradient elution systems, this affinity adsorbant could be applied to the separation of aspartase from other aspartate-utilizing enzymes, namely asparaginase and aspartate β-decarboxylase.

Kinetic Mechanism of Aspartase

As mentioned in Chapters I and II and as seen in Figures 7 and 9,

typical plots of 1/v *versus* 1/[2*S*-aspartate] for the aspartase reaction, pH $\geq$ 7.0, are 2/1 functions, curving downward at high substrate concentrations. A similar effect has been observed in the direction of fumarate amination at the highest concentration of NH_4^+ (Figure 5). Several alternatives may be offered to explain this hyperbolic behavior (Cleland, 1970).

First, one could assume that two enzymes are present which catalyze the same reaction but with different apparent K_m values. Such an assumption would give nonlinear reciprocal plots that concave downward. But if this were the case, both enzymes would have to possess very similar physical and ionic properties because Lartigue (1965) was unable to achieve separation of aspartase into two fractions with different kinetic parameters.

Since aspartase activity is enhanced by certain divalent metal ions (Wilkinson and Williams, 1961; Depue and Moat, 1961), a second alternative suggests that in the deamination reaction, 2*S*-aspartate and Mg^{2+} bind to the enzyme by a random bireactant mechanism in which the rapid-equilibrium assumption does not hold. However, all kinetic studies were conducted with saturating concentrations of Mg^{2+}, and all forms of the enzyme should have been complexed with the metal. Moreover, computer-simulated studies (Cleland, 1970) have shown that for realistic values of rate constants, random mechanisms tend to give initial velocity patterns resembling rapid-equilibrium types even if the interconversion of central complexes is not solely rate limiting. For this reason, the tendency for the double-reciprocal plots given in Figure 5 to curve downward may be unreliable as an indicator of the invalidity of the rapid-equilibrium assumption as it applies to fumarate amination.

A third possibility is that aspartase displays negative cooperativity at high substrate concentrations resulting in an increase in both K_m and V_{max}. This alternative appears most attractive since the E. aerogenes aspartase has been shown to be composed of four subunits, to exhibit homotropic substrate interactions which are pH dependent, and to be activated by certain mononucleotides under special conditions (Williams and Lartigue, 1967; Williams and Scott, 1968). Furthermore, the cooperative effects displayed by the E. coli enzyme are functions of both the Mg^{2+} and K^+ concentrations (Rudolph and Fromm, 1971; Suzuki et al., 1973).

Because the stability constant for the complex between Mg^{2+} and 2S-aspartate is relatively small (Bright, 1965), an insignificant amount of substrate should be complexed by Mg^{2+} at pH 7.0 (Bada and Miller, 1968). As a consequence, a complex of Mg^{2+} and 2S-aspartate probably does not function as a substrate in the present kinetic studies which have been conducted in the presence of 1 mM $MgSO_4$. Bright (1965) has presented evidence that a complex of Mg^{2+} and β-methylaspartate is not a substrate for the deamination reaction catalyzed by β-methylaspartase.

The initial velocity patterns in Figure 5 and 6 suggest a sequential mechanism for the aspartase reaction in the direction of fumarate amination. Since the double reciprocal plots converge on the abscissa in both cases, the apparent binding of NH_4^+ to the enzyme is independent of the concentration of fumarate, and the apparent attachment of fumarate is independent of the concentration of NH_4^+. In other words, the apparent K_m for each substrate could be equivalent to a dissociation constant in which the additional rate constants contained in the K_m are

negligibly small. Therefore, in the reverse reaction, NH_4^+ and fumarate could be in rapid equilibrium with the enzyme forms prior to the E-P-Q complex. This would further imply that in the direction of 2S-aspartate deamination, the departure of products may not be rate limiting.

Caution should be exercised, however, when using kinetic data of this type alone to establish unequivocally the location of the rate-limiting step of a sequential mechanism. According to Cleland (1970), the vertical position of the intersection point for the double-reciprocal plots is determined only by the ratio of K_d to K_m, and the observed equality of K_d and K_m may be fortuitous. Nevertheless, from these studies, a kinetic mechanism in which there is a rapid-equilibrium ordered addition of fumarate and NH_4^+ to the enzyme may be eliminated. In such a mechanism, saturation of the system with the second substrate removes any dependence of the reaction on concentrations of the first substrate by converting all of the complex between the enzyme and first substrate into the central complex (Cleland, 1970). It follows that only a concentration of the first substrate which is stoichiometric with the enzyme is needed for maximal velocity. Since that concentration is much less than the actual amount present, the double reciprocal plots obtained when the second substrate is varied at fixed levels of the first should intersect on the ordinate axis. In neither pattern of Figures 5 and 6 did an ordinate intersection occur.

From the product inhibition studies of aspartate deamination, the competitive patterns illustrated in Figures 7 and 9 indicate that both fumarate and NH_4^+ compete with 2S-aspartate for attachment to the same enzyme form, the free Mg^{2+}-enzyme complex. Moreover, Emery (1963) has

demonstrated that 2S-aspartate is a competitive inhibitor of the aspartase-catalyzed addition of hydroxylamine to fumarate. This implies that 2S-aspartate likewise should inhibit competitively the addition of NH_4^+ to fumarate. To explain the above experimental results, a random kinetic scheme rather than an ordered sequential mechanism is proposed for the aspartase reaction (Figure 19).

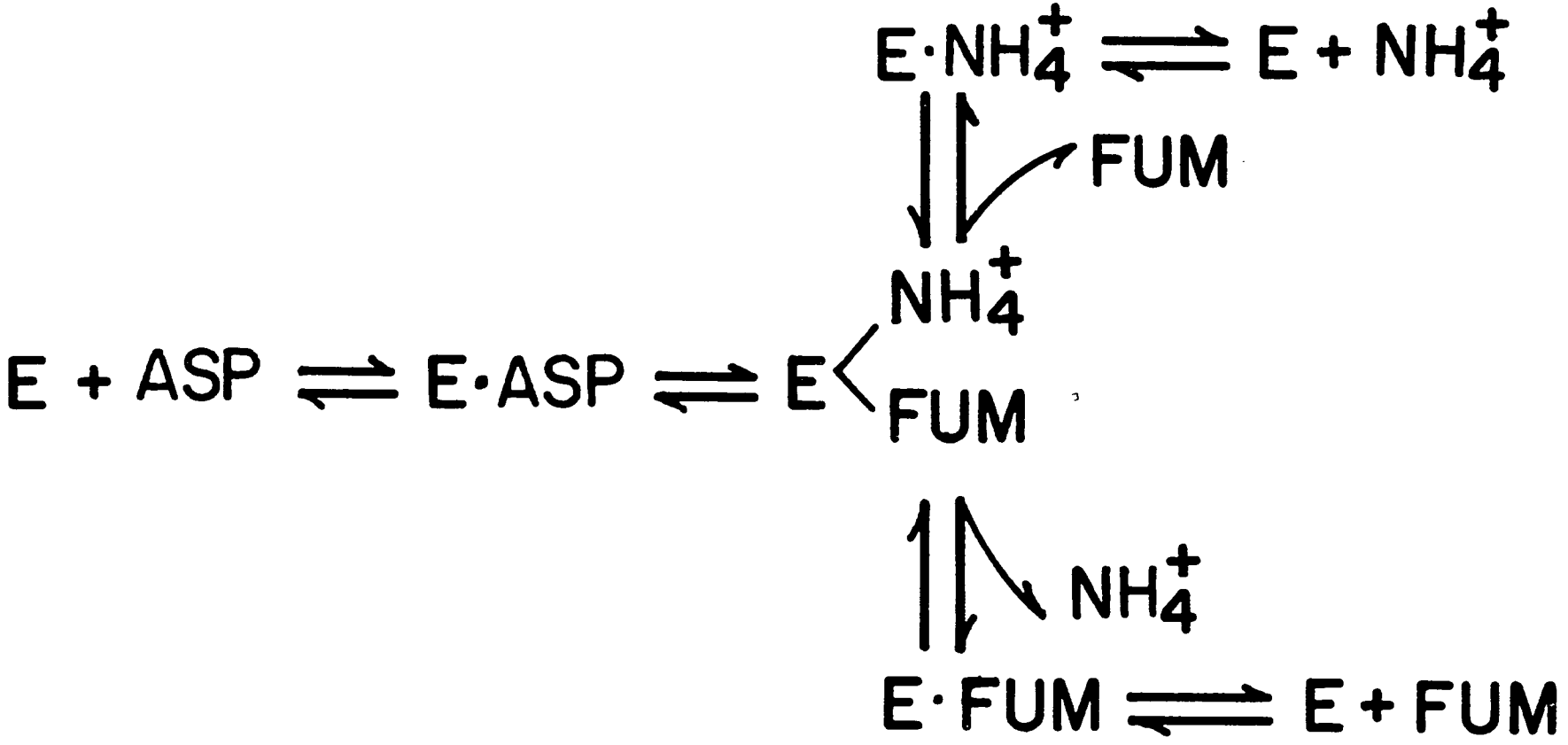
The Rate-Limiting Step

The problem of determining the rate-limiting step of the overall reaction has been investigated with forms of 2S-aspartate stereospecifically labeled with ^2H at the 3R position or with ^2H, ^3H, or ^{15}N at the α position. Confidence in the location of these isotopes in the labeled acids as well as their enrichment has been greatly enhanced by the surprisingly good agreement between the results from the mass spectral and NMR studies. When 2S,3R-[3-^{2}H]aspartate is the substrate, the label is removed during conversion of E-A to E-P-Q. Failure to observe the accompanying primary isotope effect excludes C-^{2}H bond breakage as the rate-limiting step. Likewise, removal of the corresponding proton from 2S-aspartate cannot be rate limiting. On the other hand, during the conversion of 2S-[2-^{2}H]aspartate or the ^3H-labeled form to labeled fumarate, the bond to the isotope is not broken but undergoes an sp^3-to-sp^2 hybridization. Therefore, the observation of an apparent secondary isotope effect with either labeled substrate is consistent with two alternative random mechanisms.

In the first mechanism, the reaction at the α-carbon is rate determining. If this were the situation, then the primary isotope effect

FIGURE 19

The proposed random departure of NH_4^+ and fumarate from the enzyme-products complex in the aspartase-catalyzed deamination of 2S-aspartate. It is understood that in the direction of fumarate amination, NH_4^+ and fumarate would add randomly to the enzyme.


E·NH4+ ⇌ E + NH4+
FUM
NH4+
E + ASP ⇌ E·ASP ⇌ E
FUM
NH4+
E·FUM ⇌ E + FUM

accompanying C-^{15}N bond cleavage during the deamination of 2S-[2-^{15}N]-aspartate should be detected. However, failure to observe the effect really cannot be taken as evidence against this possibility since, as was feared from the beginning, the expected kinetic isotope effect is approximately the same as the error encountered in the spectrophotometric method used herein. Yet, Bright (1964, 1965) has presented evidence that the rate-limiting step in the β-methylaspartase-catalyzed reaction is breakage of the C-N bond during conversion of the central complexes.

In the second mechanism, some step subsequent to the interconversion of the central complexes, such as product release, is rate limiting. Accordingly the observed secondary isotope effect would be an equilibrium effect which is analogous to the mechanism proposed for fumarase (Hansen et al., 1969; Rose, 1970). The absence of a primary β-isotope effect is also consistent with this mechanism even if the dissociation of the abstracted proton from the enzyme were rate-limiting. According to Rose (1970), if the proton leaves by dissociating from a conjugate acid such as an imidazolium, sulfhydryl, carboxyl, or ammonium group, the expected ^{2}H isotope effect would be quite small. All that can be said at this point is that breakage of the C-H bond is not rate limiting and the possibility exists that release of one of the products might be.

A distinction between the two alternative mechanisms can be made from the results of isotope exchange studies performed under conditions of chemical non-equilibrium and equilibrium. Since the enzyme preparation used in the ^{3}H-exchange investigation (Figure 14) was contaminated

with fumarase, the progress of deamination could be followed by the appearance of ^3H into malate as well as the formation of fumarate. Fortunately the concentration of fumarase present was sufficient to enable the hydration of fumarate to serve as an internal check on the incorporation of ^3H into 2S-aspartate since ^3H in both acids was determined by the same method. It was shown previously that, at least for the pig-heart enzyme, fumarase does not catalyze a β-hydrogen exchange which is faster than product formation nor is there a primary β-isotope effect during malate dehydration.

Reliable rates of the amination reaction can be estimated from the general rate equation derived for a rapid-equilibrium random mechanism involving one substrate and two products (Cleland, 1963). The observed rate of appearance of ^3H into aspartate is somewhat less than the rate of the reverse reaction predicted by the general rate equation. This observation suggests that the proton either is abstracted from the carbon skeleton subsequent to C-N bond breakage or departs from the enzyme following the interconversion of the central complexes. In theory, if the release of NH_4^+ or fumarate is rate limiting and the proton is not dependent on the ammonia formed for its release, then ^3H should be incorporated into 2S-aspartate at a rate faster than that allowed by the reverse reaction. Although no primary β-^2H effect was detected in the deamination reaction, it is not absolutely certain that the slow exchange of ^3H is not the result of an isotope effect. Comparative exchange studies with ^2H and ^3H would definitely clarify this point. The possibility that the abstracted proton associates with the ammonia

to form NH_4^+ following C-N bond breakage could be tested by studying the exchange of ^3H while the deamination reaction is proceeding in the presence of $[^{15}N]NH_4^+$. Equality of both exchange rates would imply that the proton is derived from the incoming NH_4^+.

If the interconversion of the central complexes of the mechanism given in Figure 19 is rate determining, the carbon and nitrogen interchange rates at chemical equilibrium should be equivalent. But the observation of a nitrogen exchange that was consistently faster than the carbon exchange under differing concentrations of reactants strongly supports the other alternative that the release of one of the products is the slow step. Apparently, after the NH_4^+ dissociates from the enzyme, the fumarate or some intermediate thereof, before being released as free fumarate, must remain bound to the enzyme long enough to be reaminated by NH_4^+ from the medium. If ^{15}N-enriched ammonium ion from the medium is represented as $^*NH_4^+$, then exchange probably occurs in the following manner:

$$E + ASP \rightleftharpoons E\cdot ASP \rightleftharpoons E\cdot FUM\cdot NH_4^+ \rightleftharpoons E\cdot FUM + NH_4^+$$

$$^*NH_4 + E\cdot FUM \rightleftharpoons E\cdot FUM\ ^*NH_4^+ \rightleftharpoons E\cdot {}^*ASP \rightleftharpoons E + {}^*ASP$$

This suggests that in the proposed random mechanism, the route by which fumarate is released last from the enzyme-product complex is favored. However, the other route whereby fumarate is released prior to NH_4^+ is also significant as indicated by the product inhibition studies and the fact that the ^{15}N exchange was only 20 to 38% faster than the ^{14}C exchange.

The location of the proton in the kinetic scheme of aspartase cannot be definitely established until detailed equilibrium exchange studies with ^{2}H and ^{3}H are performed. Nevertheless, it is of interest to speculate about what might be expected from such studies based upon the results of similar exchange studies with fumarase (Hansen, 1968; Hansen et al., 1969). When the reactant concentrations in the fumarase system approach those used in the equilibrium exchange studies with aspartase, the hydrogen exchanges more slowly than either oxygen or carbon. Thus, it has been concluded that the slow step of the reaction is proton release. Evidence from pH studies (Alberty and Pierce, 1957) and chemical modification studies with iodoacetate (Hill and Teipel, 1971) implicate at least one histidyl residue at the active site of fumarase. Although the conjugate acids of basic amino acids are known to exchange rapidly, on the order of 10^{10} M^{-1} sec^{-1} (Eigen and Hammes, 1963), the dissociation of an imidazolium group is about 1.5×10^{3} sec^{-1}. Since Hansen (1968) has interpreted the exchange of hydrogen as being neither very fast nor very slow, the proton removed from malate could very well be bound to the suspected histidyl residue.

Eigen and Hammes (1963) regard 10^{3} sec^{-1} as the upper limit for enzymatic reactions that depend upon acid-base catalysis and this is set by the maximum overall rate constant for protonation-deprontonation of catalytic groups such as imidazole. The turnover number of fumarase at pH 8 has been reported to be 245 sec^{-1} per molar active site which approximates the dissociation rate of an imidazolium moiety. Similarly, a turnover number of about 165 sec^{-1} has been found for the aspartase reaction at the optimum pH (Williams and Lartigue, 1967). Based on the

results of pH studies, Lartigue (1967) has suggested that a histidyl residue and possibly a sulfhydryl group are present at the catalytic site of the enzyme. It would not be surprising, therefore, if equilibrium exchange studies would reveal a proton exchange rate that is slower than the carbon exchange rate. If that were the case, then the kinetic mechanism would have to include a pathway in which the proton is the last product to leave the enzyme. However, to adequately explain the competitive patterns obtained in the product inhibition studies, the mechanism also would have to show the departure of the proton as being random, even though those routes whereby the proton is released last would be favored. As can be seen, the mechanism would become quite complex.

Chemical Mechanistic Implications

In the deamination reaction catalyzed by β-methylaspartase, the evidence presented by Bright (1964) has strongly suggested that the interconversion of the central complexes is rate-limiting. Accordingly, isotope effect and non-equilibrium exchange studies have been used to establish that the elimination probably proceeds by a carbanion mechanism. Moreover, the non-enzymatic deamination of aspartate (pH 6-12; 116.3°) has been shown to occur by a similar mechanism (Bada and Miller, 1970). Therefore, it was hoped that the mechanism of the deamination reaction as catalyzed by aspartase could also be determined and compared with the non-enzymatic case to test the idea that during their evolution, enzymes more or less took advantage of reaction mechanisms already present in the environment.

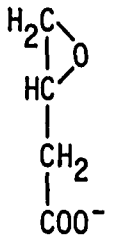
Unfortunately, it has been shown that the rate-limiting step of the aspartase reaction probably occurs subsequent to the bond-breaking steps

in the interconversion of the central complexes. The data from the isotope effect and non-equilibrium exchange studies, though substantiating the location of the rate-limiting step, cannot be used to distinguish between possible organic mechanisms. Consequently, other approaches will have to be used to study this problem.

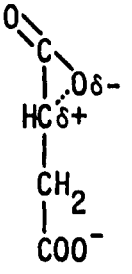
One that readily comes to mind is the use of transition state analogs as inhibitors. Such analogs are compounds which resemble the structure assumed by the real substrate as it passes through the transition state of the reaction. In terms of transition state theory as it applies to enzymes (Wolfenden, 1972), catalysis is effected because the enzyme binds the transition-state structure of the substrate much more tightly than it binds the substrate. It follows that a compound mimicking the transition-state structure of the substrate would be a much more potent competitive inhibitor than one resembling the corresponding ground-state structure.

In an earlier report, Dougherty et al. (1972) using data from initial velocity, product inhibition, isotope effect, and non-equilibrium exchange studies favored an enzymatic mechanism in which the slow step was C-N bond breakage followed by removal of the proton from aspartate. The somewhat reduced secondary isotope effect as commonly found for model systems was due either to the reaction at the α carbon not being entirely rate limiting or to the participation of a carboxylate group from the substrate itself. Another possibility was the participation of some nucleophile from the enzyme during or immediately following breakage of the C-N bond (Richards, 1970). It was further stated that the nonenzymatic deamination of an amino acid with nitrous acid was thought to proceed through the formation of a transient α-lactone intermediate leading to retention of

configuration in the α-hydroxyl acid produced (Neuberger, 1948; Brewster et al., 1950). If in the aspartase reaction, an α-lactone is indeed a transition state intermediate, then a good transition state analog to test initially might be potassium 3,4-epoxybutonate, the structure of which is:



3,4-epoxybutonate
(K^+ salt)



proposed α-lactone intermediate

If this compound inhibits competitively the deamination of 2S-aspartate, the observation of a K_i several orders of magnitude less than the apparent K_d or K_m for the substrate would imply that such a carbonium-ion intermediate is likely. Pertinently, a carbonium-ion mechanism has been proposed for the fumarase mechanism, although the analogous non-enzymatic elimination of water from malate is thought to proceed through a carbanion intermediate (Bada and Miller, 1970).

Until more explicit information can be obtained which would suggest otherwise, the most likely mechanism for the aspartase reaction probably involves a carbanion intermediate similar to that proposed by Lartigue (1965). In such a mechanism, the pair of free electrons on a group such as an imidazole could attract the β hydrogen of the substrate thereby weakening the C-H bond. The proton would eventually be abstracted and the

resulting carbanion could be stabilized through the β carboxyl group which might be complexed with Mg^{2+} at the active site. The carbanion would then collapse into the olefin, expelling the amino group.

Also a concerted mechanism of the following type may be possible. A nucleophile such as $-S^-$ may initially attack the α-carbon to form a transition state of considerable olefin character. The nucleophile then would combine with the proton and depart from one face of the fumaric acid while the amino group departed from the other (Hanson and Havir, 1972).

At this point, it should be obvious that before any conclusions can be drawn concerning the chemical mechanism of an enzyme-catalyzed reaction, the kinetic scheme with the accompanying rate-limiting step must be determined or substantiated through the use of all of the kinetic techniques described in this dissertation. The results obtained from any one of them could be quite misleading when considered alone. In the case of the aspartase reaction, the concurrence of data from initial velocity, product inhibition, isotope effect, and non-equilibrium exchange studies is not sufficient to determine the validity of the rapid-equilibrium assumption. In fact, this is possible only when the above results are considered together with the data from the equilibrium-exchange investigations. Since the rate-determining step of the aspartase reaction is probably the release of one of the products and not the interconversion of the central complexes, the value of kinetic isotope effects as direct probes of the chemical mechanism of this reaction is severely reduced.

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APPENDIX I

TABLE OF ABBREVIATIONS

A	Absorbance
A	2S-Aspartate
AMP	Adenosine monophosphate
ASP	2S-Aspartate
cm	Centimeter(s)
cpm	Counts per minute
Ci	Curie(s)
DEAE	Diethylaminoethyl
EDTA	Ethylenediaminetetraacetic acid
F	Fraction of isotopic equilibrium
FUM	Fumarate
g	Gram(s)
g	Gravity
GOT	Glutamate-oxaloacetate transaminase
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
Hz	Hertz
IMP	Inosine monophosphate
l	Liter(s)
M	Molar
mCi	Millicurie(s)
m/e	Mass-to-charge ratio
mequiv	Milliequivalent(s)
mg	Milligram(s)

MHz	Megahertz
min	Minute(s)
ml	Milliliter(s)
mM	Millimolar
N	Normal
NAD^+	Nicotinamide-adenine dinucleotide (oxidized form)
nm	Nanometer(s)
NMR	Nuclear Magnetic Resonance (Spectroscopy)
P	NH_4^+
ppm	Parts per million
Q	Fumarate
t	Time
Tris	Tris(hydroxymethyl)aminomethane
w	Weight
α	Carbon 2 of 2S-aspartate
β	Carbon 3 of 2S-aspartate
μCi	Microcurie(s)
μl	Microliter(s)
μM	Micromolar(s)

APPENDIX II

TABLE OF KINETIC TERMINOLOGY

A, B	Substrates designated in the order in which they add to the enzyme in a sequential mechanism
P, Q	Products designated in the order in which they are released from the enzyme in a sequential mechanism
E	Free enzyme or free enzyme saturated with a metal cofactor
E-A-B	Central complex of enzyme and substrates
E-P-Q	Central complex of enzyme and products
[A], [P]	Reactant concentrations
∞	Infinite reactant concentration
K_m or K	Michaelis constant
K_d	Dissociation constant
K_i	Inhibition constant
V_{max} or V	Maximum velocity
V_1 and v_1	Maximal and initial velocities of the forward reaction, respectively
V_2 and v_2	Maximal and initial velocities of the reverse reaction, respectively
k	Rate constant of the reaction under consideration

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